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Parent et al.

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(54) **CULTURE MEDIUM FOR MYOBLASTS, PRECURSORS THEREOF AND DERIVATIVES THEREOF**

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**A61K 35/12** (2015.01)

(52) **U.S. Cl.**

CPC ..... **C12N 5/0658** (2013.01); **A61K 35/12** (2013.01); **C12N 2500/25** (2013.01); **C12N 2500/84** (2013.01); **C12N 2500/99** (2013.01); **C12N 2501/105** (2013.01); **C12N 2501/11** (2013.01); **C12N 2501/115** (2013.01); **C12N 2501/135** (2013.01)

(58) **Field of Classification Search**

CPC combination set(s) only.

See application file for complete search history.

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#### **ABSTRACT**

This application relates to a serum-free culture medium that enables the proliferation of cell of the myogenic lineage while maintaining their ability to differentiate into functional muscle cells. Also contemplated herewith are method of culturing cells of the myogenic lineages and uses of the cultured cells for the treatment or the alleviation of symptoms of a muscular deficiencies.

**22 Claims, 11 Drawing Sheets**

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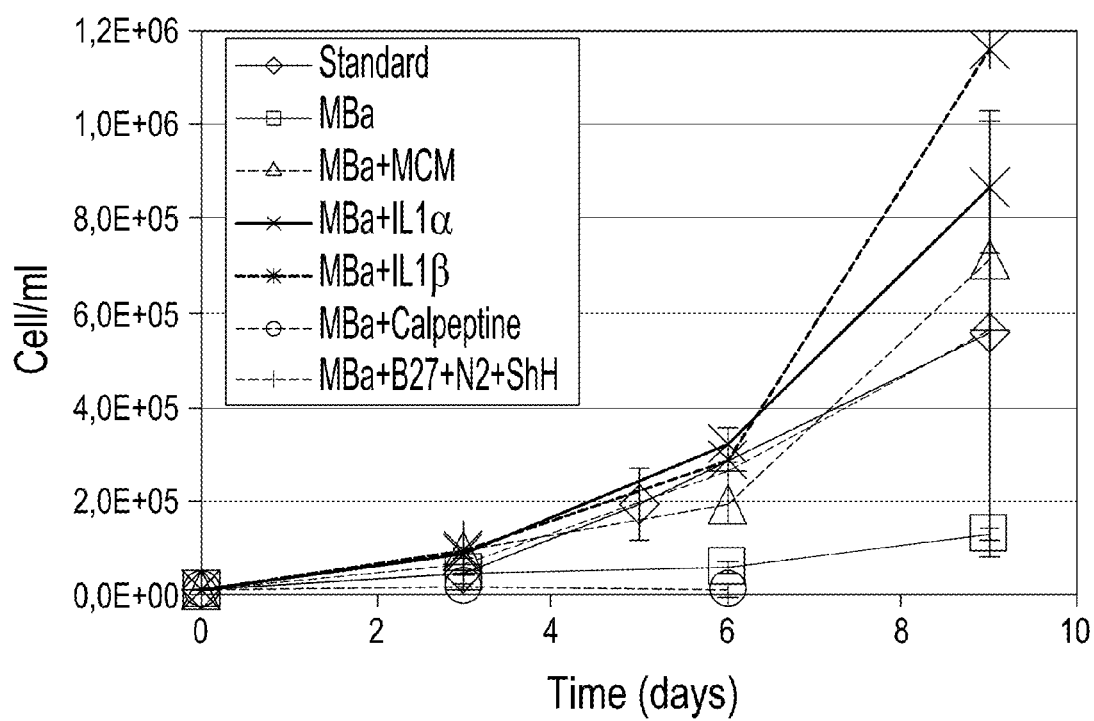


FIG. 1

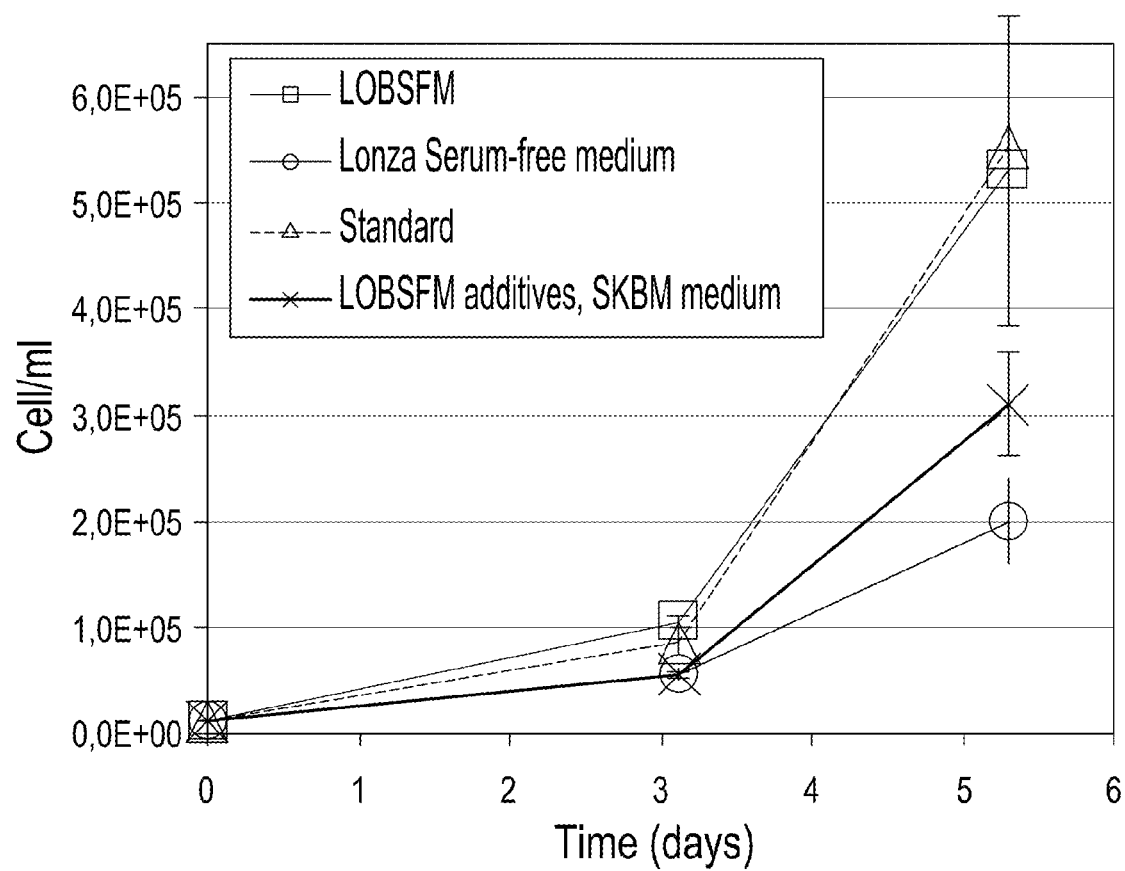


FIG. 2

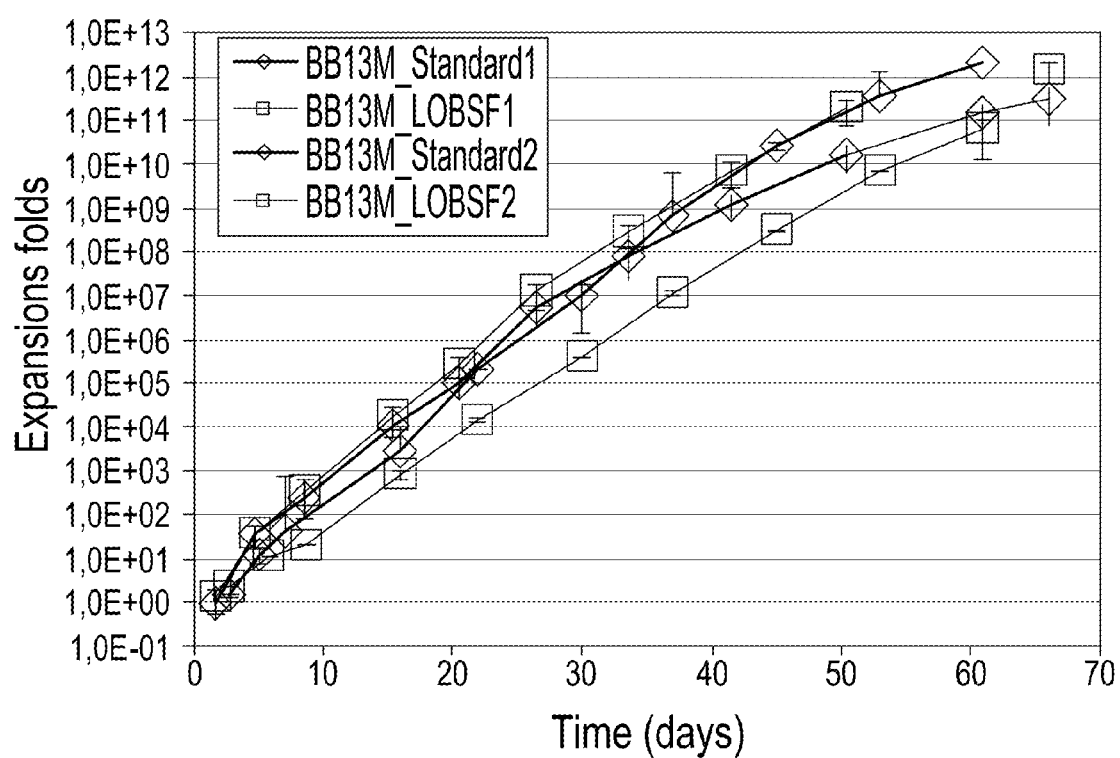


FIG. 3

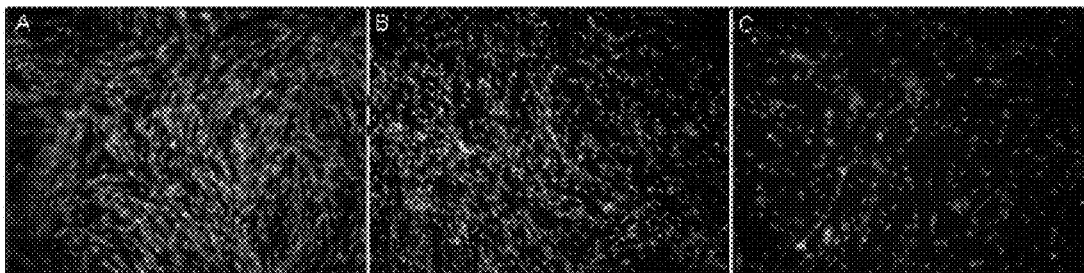


FIG. 4A

FIG. 4B

FIG. 4C

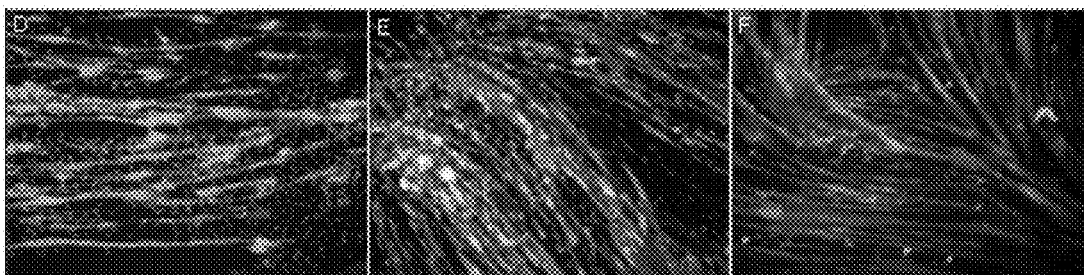


FIG. 4D

FIG. 4E

FIG. 4F

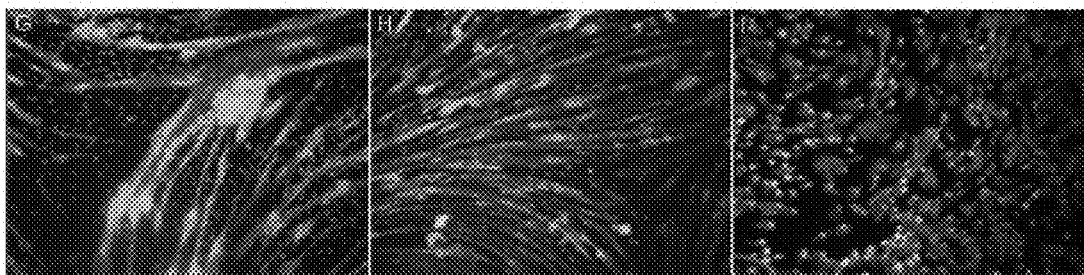


FIG. 4G

FIG. 4H

FIG. 4I

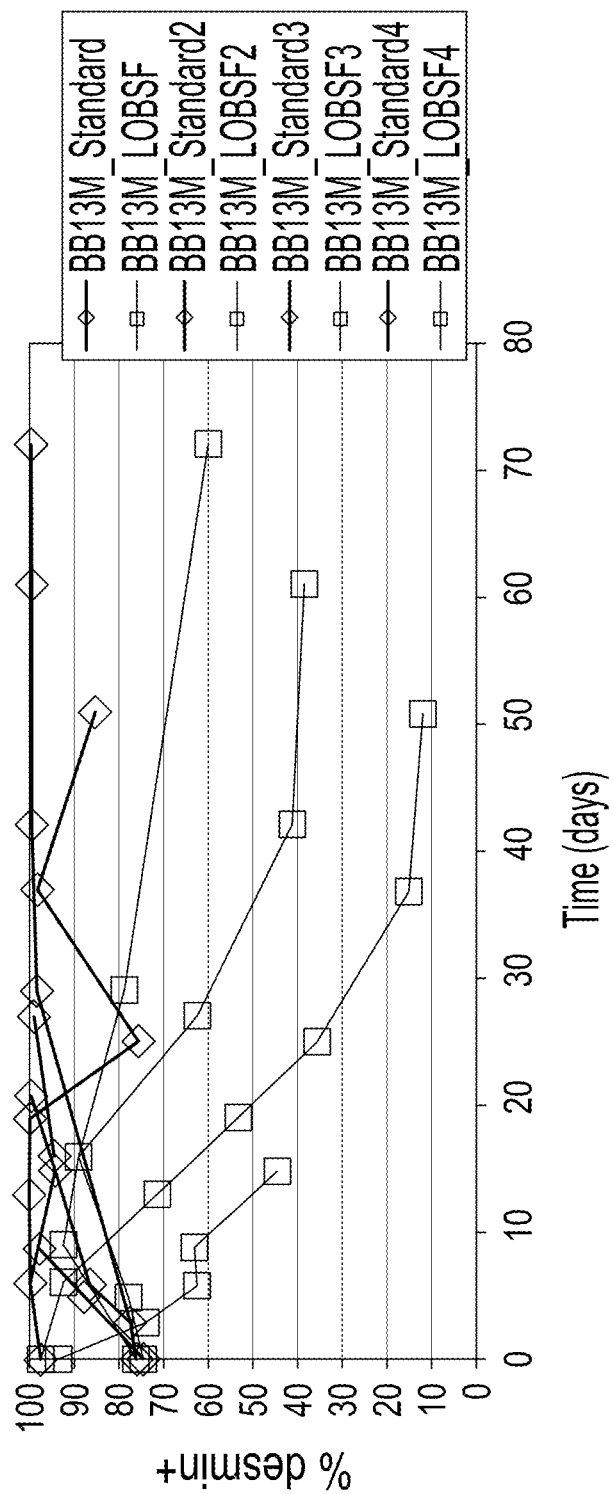


FIG. 5A

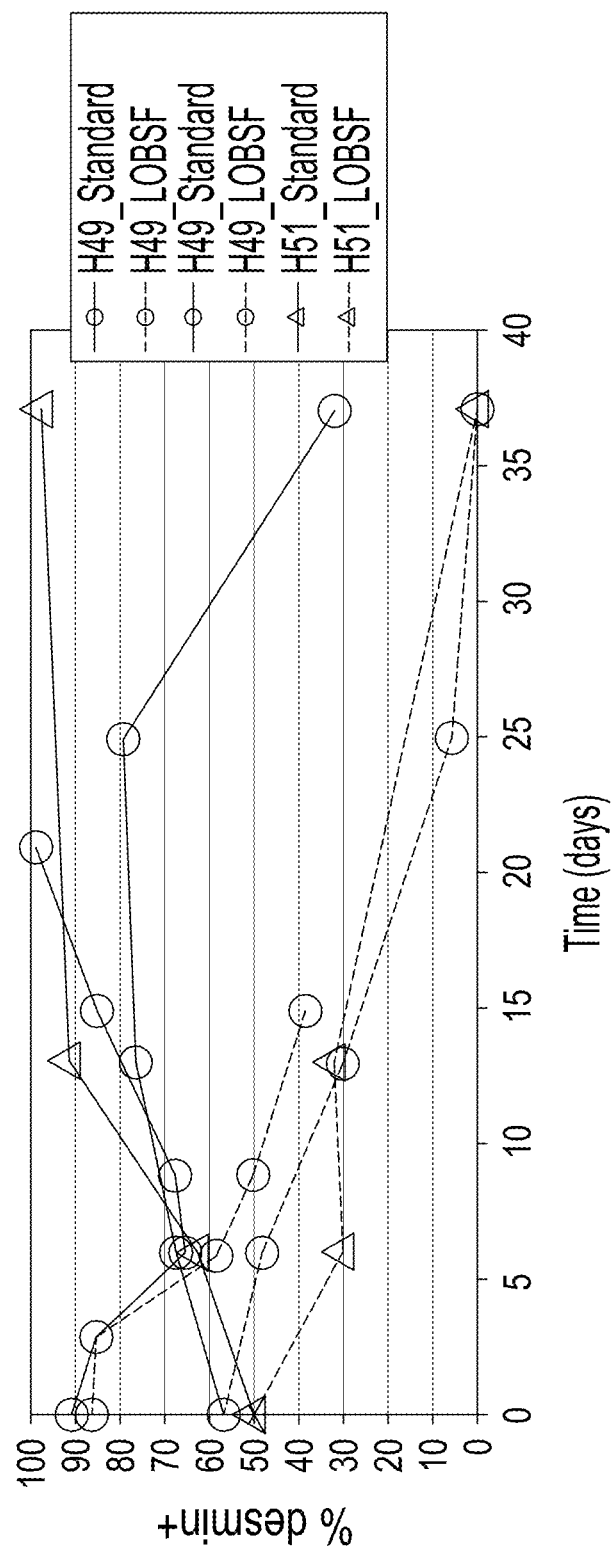


FIG. 5B



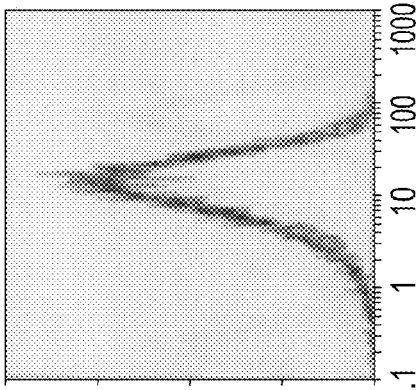


FIG. 6A

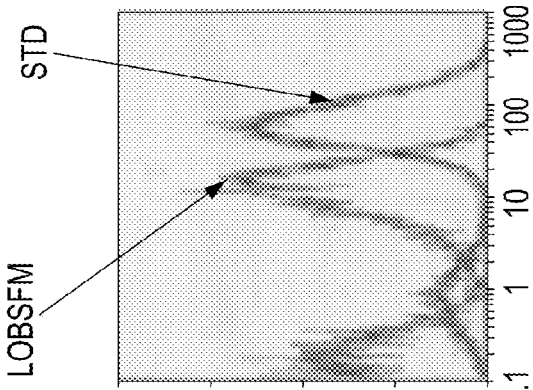


FIG. 6B

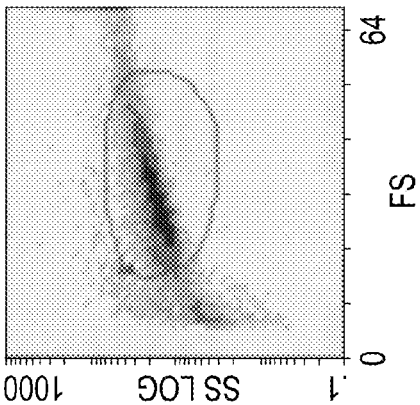


FIG. 6C

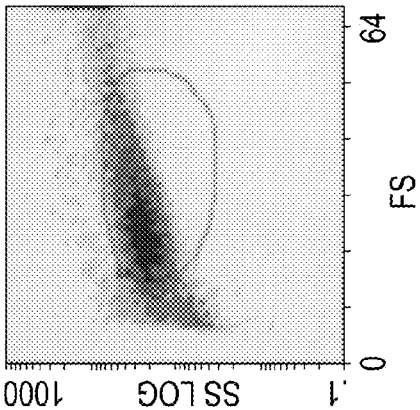


FIG. 6D

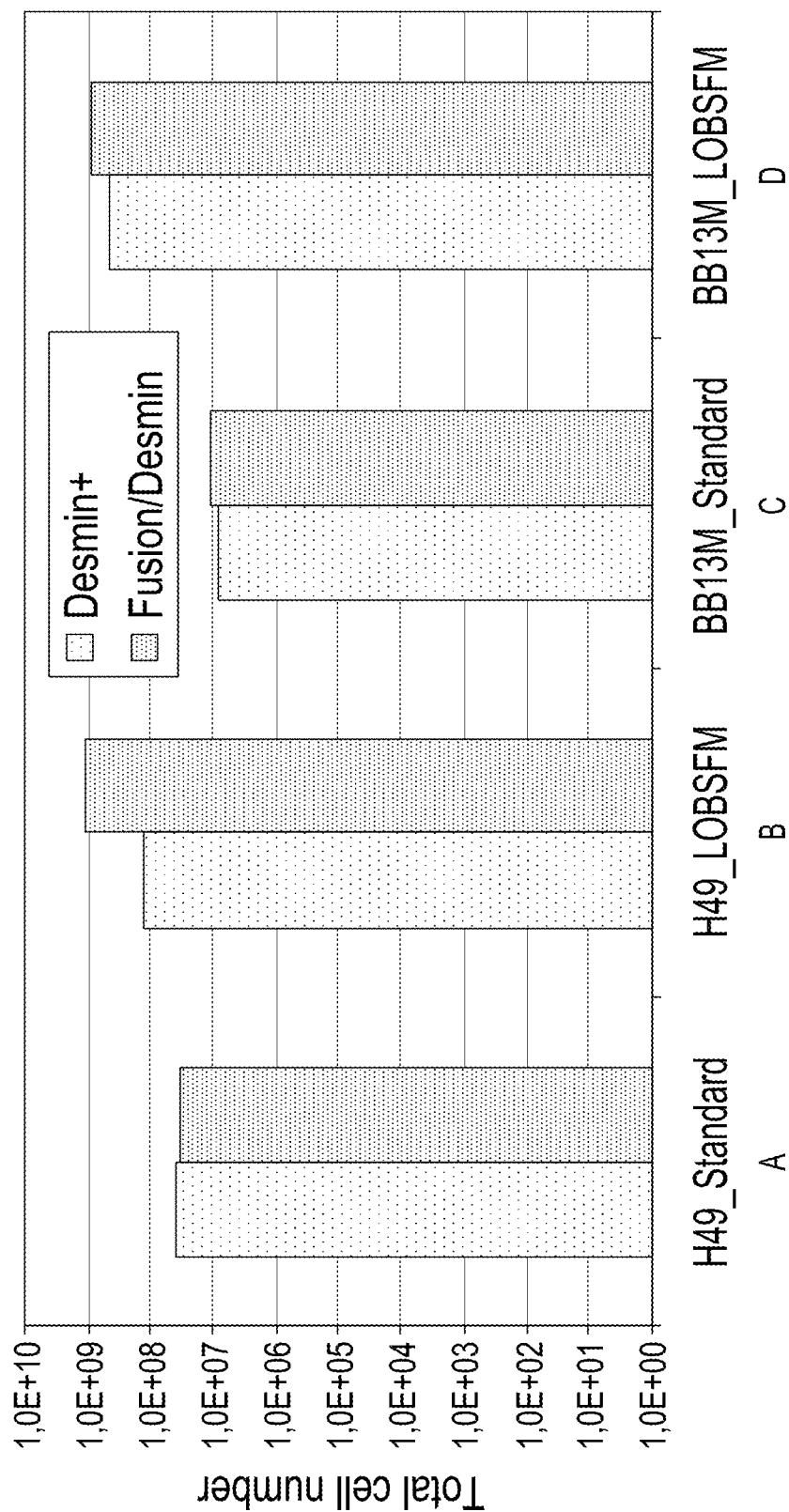


FIG. 7

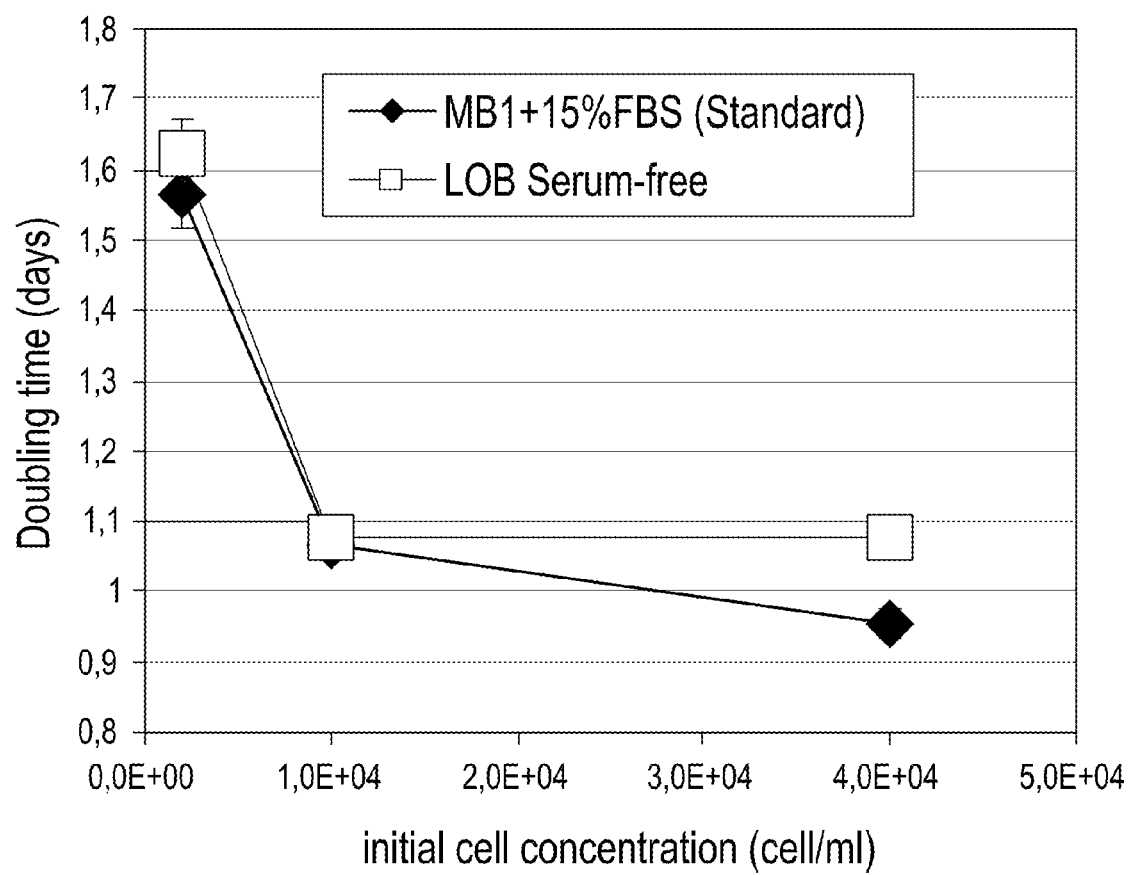


FIG. 8

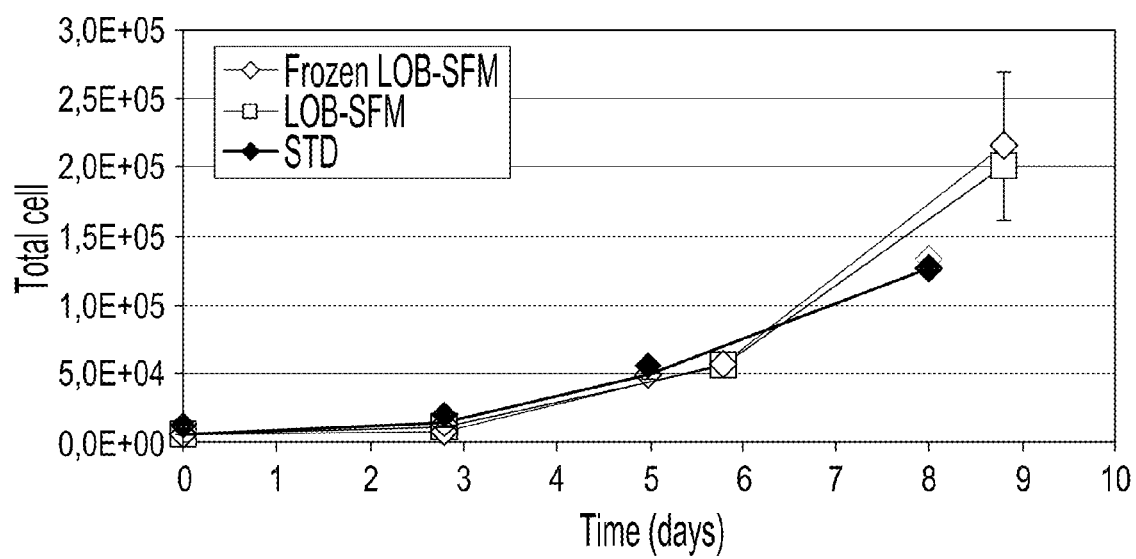


FIG. 9

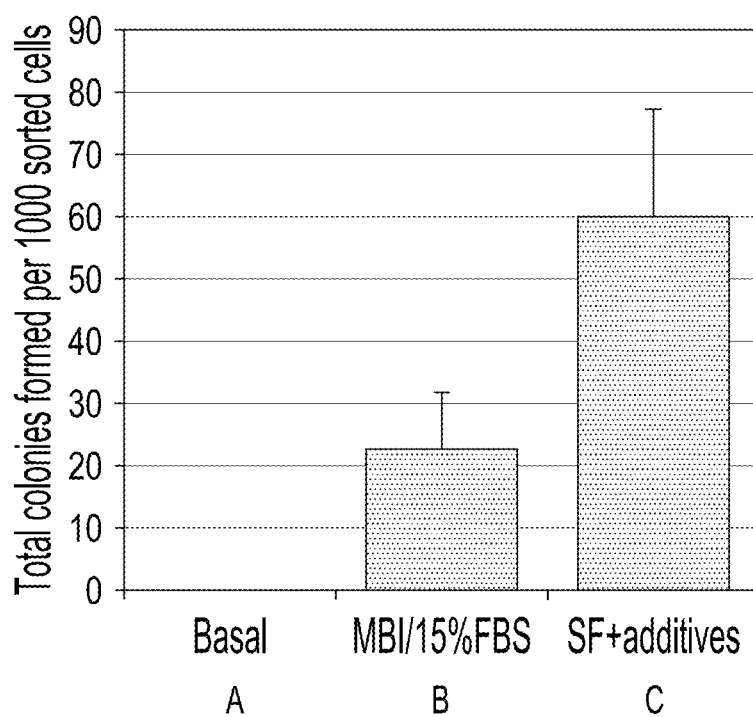


FIG. 10

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# **CULTURE MEDIUM FOR MYOBLASTS, PRECURSORS THEREOF AND DERIVATIVES THEREOF**

## **CROSS-REFERENCE TO RELATED APPLICATIONS**

This is application claims priority from U.S. provisional application Ser. No. 61/098,964 filed on Sep. 22, 2008 and incorporated herewith by reference in its entirety.

## **STATEMENT REGARDING SEQUENCE LISTING**

The Sequence Listing associated with this application is provided in text format in lieu of a paper copy, and is hereby incorporated by reference into the specification. The name of the text file containing the Sequence Listing is 920116\_401USPC\_SEQUENCE\_LISTING.txt. The text file is 36 KB, was created on Mar. 21, 2011, and is being submitted electronically via EFS-Web.

## **BACKGROUND OF THE INVENTION**

Duchenne muscular dystrophy (DMD), with a prevalence of 1 boy out of 3500, is the most widely spread genetic disease (Voisin, 2004). This disease is characterized by the progressive weakening of muscles, resulting in muscular necrosis and fibrosis (Emery, 2003). An essential protein for muscle fibers stability, dystrophin, is missing or defective in muscle fibers of DMD patients. Dystrophin is a 427-kDa protein which connects the actin cytoskeleton to the cellular membrane (Bogdanovich et al. 2004). The function of this protein is to maintain the stability of the cellular membrane in order to support the stresses induced during muscular contractions (Carpenter et al. 1990). The absence of dystrophin increases the vulnerability of muscle fibers during their contraction. Consequently, constant muscular repair will be necessary, which will result in the premature senescence of myoblasts.

Cellular therapy, a therapy under development to counter DMD, has shown significant therapeutic effect in several studies in mice (Chen, Li et al. 1992) and humans (Gussoni et al. 1992; Huard et al. 1992; Huard et al. 1994; Skuk et al. 2006; Skuk et al. 2007). This curative approach is regarded as a promising treatment (Skuk and Tremblay, 2000). Presently, cellular therapy consists in injecting human myoblasts in the muscles of the DMD patients. The healthy myoblasts fuse to muscle fibers of the patients and partially restore the expression of dystrophin. This expression increases the strength of the treated muscle and restores at least partially their functionality, hence improving significantly the patient's quality of life. Nevertheless, some difficulties remain to be surmounted, of which the immunizing response against the injected myoblasts, the absence of fusion of myoblasts with undamaged fibers as well as the poor migration of the myoblasts in the muscle tissue. Moreover, the culture medium presently used for myoblast expansion contains blood serum and the production processes presently used are not adequate for the production of the large number of myoblasts that would be required to treat the whole muscle mass of a patient. Constant and rapid progresses are made on these fronts, through better understanding of myoblast and myogenic cell biology, transplantation and migration, the development of protocols to alleviate immuno-rejection, and the identification of alternative cell sources, e.g. pluripotent stem cells

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(Skuk et al. 2002). Cellular therapy therefore seems to be on the verge of being the very first accepted therapy against DMD.

In addition to the treatment of DMD, myoblast injections are also used for the treatment of myocardial infarction and for urethral sphincter insufficiency. Indeed, the grafts of myoblasts significantly improve the contractility as well as the viability of cardiac tissues and allows 41% restoration of the normal sphincter contractility (Yiou et al. 2004; Kahn, 2006).

The nutritional needs of in vitro cultured cells comprise a vast range of molecules, of which several are found in a basal medium, which contains the essential components necessary to the cellular metabolism and osmotic balance maintenance: salts, amino-acids, vitamins, sugars, lipids, trace elements, antioxidants and pH buffer (Ham and McKeehan 1979; Butler, 1991; Mather and Barnes 1998; Davis, 2002; Naomoto et al. 2005). The basal medium must be selected considering its compatibility with the type of cells to be cultured, as well as its performance with regards to predetermined responses of interest. The role of the basal medium can therefore either be to support proliferation, differentiation or quiescence of a given cell population (Zimmerman et al. 2000). Basal media known to support myoblast expansion are DMEM, F12, RPMI1640 (Goto et al. 1999) and MCDB120 (Ham et al. 1988).

The medium generally used to culture myoblasts in vitro (standard medium, STD) is MCDB120 supplemented with 15% fetal bovine serum (FBS), 10 ng/ml basic fibroblast growth factor (bFGF), 0.39 µg/ml dexamethasone and 0.5 mg/ml bovine serum albumin (BSA). Serum is an additive which allows the non-specific proliferation of a vast range of cell types. It is prepared from plasma, the liquid fraction of blood, from which clotting factors were removed. The principal sources of sera for cell culture are bovine fetal blood (FBS), calf (CS), horse (HS) or human. FBS is the most widely used. Its functions are multiple: adhesion of the cells to a solid surface (via adhesion molecules such as fetuin, fibronectin, vitronectin), growth stimulation (growth factors, GF; cytokines; hormones), protection (antioxidants, antitoxins, proteins), buffer and nutrition. However, serum composition is not completely resolved since it is a very complex fluid, containing at least 3 000 different proteins (Omenn, 2005). Another point to consider is the quickly rising cost of serum, due to an increasing demand (500-600\$/L) (Davis, 2002). Moreover, serum requires extensive quality control, it contains proliferation inhibitors, its composition varies from batch to batch, and it hinders the purification of cell culture products. Serum can also be contaminated by viruses, bacteria and prions (Jayme et al. 1988; Freshney, 2000). It is therefore advantageous to replace serum by a mixture of defined components that would not present similar problems. Serum-free media allow a better control of cell proliferation and differentiation and reduce the risk of contamination (Zimmerman et al. 2000). However, serum-free media are much more specific than serum-containing media, often only supporting the growth or differentiation of the cell type for which it has been developed. The corollary of this is therefore that most cell lineage will require the development of their own serum-free medium.

Although serum-free medium formulations for myoblast culture have previously been reported (Ham et al. 1988) or are commercially available (Skeletal muscle cell medium BUL-LETKIT™, CC-3160 from Lonza), the extent and rate of myoblast proliferation in these media remains much lower than in serum containing media. To this date, Applicant is not aware of any effective serum-free medium for myoblast expansion.

Consequently, the development of a safer, serum-free culture medium that would efficiently support the expansion of myoblasts and their precursors would significant and timely contribute to the advent of cellular therapy based on the use of these cells.

### SUMMARY OF THE INVENTION

The present invention relates to a culture medium for cells of the myogenic lineage that is free of serum and/or any other undefined supplement. This serum-free culture medium allows the cells to proliferate at a rate that is similar to the one of cells cultured in a serum-containing medium. The present invention also relates to a method for culturing cells in vitro where the serum-free medium is used. The use of the cells cultured in the serum-free media in cellular therapy for muscle-associated conditions is also contemplated.

According to a first aspect, the present invention provides a culture medium for a cell of the myogenic lineage. In an embodiment, the culture medium comprises a basal medium and a cytokine or a combination of cytokines. Preferably, the culture medium is free of serum and allows the proliferation of the cell of the myogenic lineage at a similar rate than another cell of the myogenic lineage cultured in a standard medium containing serum. In an embodiment, the cell of the myogenic lineage is at least one of a muscular stem cell, a myoblast and a myoblast-derived cell. In another embodiment, the muscular stem cell can differentiate into a myoblast. In yet another embodiment, the myoblast-derived cell is at least one of a muscle cell, a satellite cell and a myocyte. In still another embodiment, the cell of the myogenic lineage is derived from a biopsy, is a mammalian cell and/or is a human cell. In an embodiment, the basal medium is at least one of Dulbecco's modified Eagles's medium (DMEM), advanced DMEM, BIOGRO™, SKGM®, Ham's F10, Ham's F12, Iscove's modified Dulbecco's medium, neurobasal medium, RPMI 1640 and MCDB120 medium. In another embodiment, the basal medium is a combination of Ham's F12, RPMI 1640 and MCDB120 and, in a further embodiment, the proportion of Ham's F12, RPMI 1640 and MCDB120 is about 1:1:1. In an embodiment, the cytokine is a human cytokine. In still another embodiment, the cytokine is a recombinant cytokine. In yet another embodiment, the cytokine is at least one of a growth factor (GF) and an interleukin. In still another embodiment, the growth factor is at least one of a fibroblast growth factor (FGF), an epidermal growth factor (EGF) and an insulin-like growth factor (IGF). In yet another embodiment, the concentration of FGF in the culture medium is between about 1 and 20 ng/ml. In an embodiment, the FGF is at least one of FGF-1, FGF-2, FGF-4, FGF-5, FGF-6, FGF-7, FGF-8 and FGF-9. In still another embodiment, the concentration of EGF in the culture medium is between about 1 and 20 ng/ml. In yet another embodiment, the concentration of IGF in the culture medium is between about 1 and 50 ng/ml. In an embodiment, the IGF is at least one of IGF-1 and IGF-2. In still another embodiment, the concentration of interleukin in the culture medium is between about 0.1 to 20 ng/ml. In an embodiment, the interleukin is at least one of IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, IL-16, IL-17, IL-18, IL-19, IL-20, IL-21, IL-22, IL-23, IL-24, IL-25, IL-26, IL-27, IL-28, IL-29, IL-30, IL-31, IL-32 and IL-33. In yet another embodiment, the culture medium further comprises a supplement and, in yet another embodiment, the supplement is at least one of: i) a combination of insulin (at a concentration, for example, of 0.5 mg/ml), transferrin (at a concentration, for example, of 5 mg/ml) and selenite (at a concentration, for example, of 0.52 µg/ml)

(ITS); ii) B27 medium supplement; iii) a combination of dexamethasone, insulin, EGF, fetuin and albumin; and iv) a combination of dexamethasone, bFGF, albumin and insulin. In still another embodiment, the concentration of ITS in the culture medium is between about 0.5 and 2.5% v/v. In yet another embodiment, the culture medium further comprises a lipid and, in still another embodiment, the lipid is at least one of arachidonic acid, cholesterol, DL- $\alpha$ -tocopherol acetate, linoleic acid, linolenic acid, myristic acid, oleic acid, palmitoleic acid, palmitic acid and stearic acid. In still another embodiment, the concentration of the lipid in the culture medium is between about 0.5 and 2.5% v/v.

In a second aspect, the present invention provides an in vitro method of culturing a cell of the myogenic lineage. The method can comprise contacting the cell of the myogenic lineage with the culture medium described herein thereby culturing said cell. In an embodiment, the cell of the myogenic lineage is at least one of a muscular stem cell, a myoblast and a myoblast-derived cell. In a further embodiment, the muscular stem cell can differentiate into a myoblast. In yet another embodiment, the myoblast-derived cell is at least one of a muscle cell, a satellite cell and a myocyte. In an embodiment, the method is performed for at least an hour, for at least a day, for at least a week or for at least a month. In an embodiment, the method reduces the lag phase of the cell of the myogenic lineage with respect to the lag phase of a cell of the myogenic lineage cultured in another serum-free media. In another embodiment, the method enables the long term expansion of the cell of the myogenic lineage. In still another embodiment, the initial concentration of the cell of the myogenic lineage in the culture medium is 10 000 cells/mL. In yet another embodiment, the method enables the cell of the myogenic lineage to retain its ability to form a myotube.

In a third aspect, the present invention provides a method of treating or alleviating a muscular deficiency in a subject in need thereof. In an embodiment, the method comprises contacting a cell of the myogenic lineage with the culture medium described herein; and implanting the cell previously obtained in the subject; thereby treating or alleviating the muscular deficiency in the subject. In an embodiment, the method comprises providing a cell of the myogenic lineage cultured by the method described herein and implanting the cultured cell obtained in the subject; thereby treating or alleviating the muscular-associated condition in the subject. In an embodiment, the subject is a human. In another embodiment, the muscular-deficiency is at least one of Duchenne muscular dystrophy, myocardial infarction, urethral sphincter insufficiency.

In a fourth aspect, the present invention provides the use of a cell of the myogenic lineage cultured in the culture medium described herein or prepared by the method described herein for the treatment or the alleviation of symptoms of a muscular deficiency in a subject. In an embodiment, the subject is a human. In another embodiment, the muscular deficiency is associated with at least one of Duchenne muscular dystrophy, myocardial infarction, urethral sphincter insufficiency.

In a fifth aspect, the present invention provides a cell of the myogenic lineage cultured in the culture medium described herein or prepared by the method described herein for the treatment or the alleviation of symptoms of a muscular deficiency in a subject. In an embodiment, the subject is a human. In another embodiment, the muscular deficiency is associated with at least one of Duchenne muscular dystrophy, myocardial infarction, urethral sphincter insufficiency.

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## BRIEF DESCRIPTION OF THE DRAWINGS

Having thus generally described the nature of the invention, reference will now be made to the accompanying drawings, showing by way of illustration, a preferred embodiment thereof, and in which:

FIG. 1 compares the expansion of human myoblasts cultured in various media ( $\diamond$ , standard (STD) medium;  $\square$ , first generation serum-free medium (MBa);  $\Delta$ , MBa supplemented with macrophage conditioned medium (MCM); x, MBa supplemented with interleukin 1 $\alpha$  (IL-1 $\alpha$ ); \*, MBa supplemented with IL1 $\beta$ ;  $\circ$ , MBa supplemented with calpeptine; l, MBa supplemented with B27 supplement, N2 supplement and sonic hedgehog (ShH) during the course of a nine-day culture.

FIG. 2 compares the expansion of human myoblasts cultured in various media ( $\square$ , optimized serum-free medium (LOBSFM);  $\circ$ , Lonza serum-free medium composed of the basal medium SKBM<sup>®</sup> supplemented with BULLETKIT<sup>™</sup> additives;  $\Delta$ , standard medium; x, SKBM<sup>®</sup> medium supplemented with the LOBSFM additives for five days.

FIG. 3 compares the long-term expansion of BB13 cells in various media ( $\diamond$ , standard medium, duplicates 1 and 2;  $\square$ , LOBSFM, duplicates 1 and 2) expressed in expansion folds in function of time (days).

FIG. 4 shows micrographs of immunostained cells that proliferated in LOBSFM (A to F) and STD medium (G to I) with cell nuclei represented as dots; desmin represented as elongated structures in panels A-C, E and I, myosin heavy chain represented as elongated structures in panels D, F, G, H. The cells were cultured either for one week (D and G), two weeks (A), four weeks (B, E, H) or six weeks (C, F, I). Cells in boxes D to H were fixed and immunostained after a three-day incubation in the differentiation medium.

FIG. 5 illustrates the proportion of desmin-expressing cells from independent experiments for (A) BB13 cells in STD medium ( $\diamond$ ) or LOBSFM ( $\square$ ); and (B) H49 ( $\circ$ ) or H51 ( $\Delta$ ) cells in STD medium (solid line) or LOBSFM (broken line).

FIG. 6 shows flow cytometry results of a four weeks myoblast culture. (A) BB13 cellular counts against intensity for NCAM<sup>+</sup> cells cultured in LOBSFM (98.8%) or STD medium (96.9%). (B) H49 cellular counts against intensity for cells cultured in LOBSFM (left curve) or standard medium (right curve) as indicated by the arrows. (C) Side scatter against forward scatter for cells cultured in standard medium. (D) Side scatter against forward scatter for cells cultured in LOBSFM medium. The gated area in (C) and (D) represent cells analyzed for fluorescence.

FIG. 7 illustrates the number of desmin-positive cells before (pale gray) and after (dark grey) fusion generated after a 25-day culture of H49 cells in STD medium (A) or LOBSFM (B) and of BB13 cells in STD medium (C) or LOBSFM (D).

FIG. 8 illustrates the doubling time (in days) of BB13 cells cultured in standard ( $\diamond$ ) or LOBSFM ( $\square$ ) medium in function of initial cellular concentration.

FIG. 9 compares the expansion of cells in function of days in culture of BB13 cells in standard ( $\diamond$ ), fresh LOBSFM ( $\square$ ), or frozen/thawed LOBSFM ( $\Delta$ ) medium.

FIG. 10 illustrates the total colonies formed per 1 000 of FACS sorted mouse myogenic progenitor cells (gated for nucleated, live cells using Hoechst<sup>+</sup>, and PI<sup>-</sup>, and stained with CD31<sup>-</sup>, CD45<sup>-</sup>, sca1<sup>-</sup>, and alpha7<sup>+</sup> antibodies were used) in (A) basal medium, (B) standard medium and (C) LOBSFM medium.

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## DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

Broadly, the present application relates to a culture medium that enables the in vitro expansion of cells of the myogenic lineage (such as muscular stem cells, myoblasts and myoblast-derived cells) that is free of serum. The main advantages of this serum-free culture medium is its safety, the elimination of growth inhibitors contained in serum, a more consistent lot to lot medium composition, and easier approbation by regulating authorities for its use in clinical trials (Freshney 2005). The proposed culture medium allows cells to proliferate rapidly and to high concentration without an adaptation period. That period is often necessary to slowly adapt the cells by reducing progressively serum concentration. It also permits the rapid proliferation of cells to a high concentration if the cells are seeded at a low concentration (such as 10 000 cells/ml). Further, the serum-free medium does not stimulate the differentiation and fusion of myoblasts into myotubes. However, the serum-free medium allows cultured myoblasts to maintain their capacity to form myotubes in vitro when transferred into a differentiating medium. Consequently, it is contemplated that the cells cultured in the medium retain their ability to fuse to muscle fibers in vivo.

According to one aspect, the culture medium provided herein is for a cell of the myogenic lineage. As used herein, the term "cell of the myogenic lineage" refers to a cell capable of expressing genes and proteins specific to myoblasts or to a cell having a myoblastic phenotype. Myoblast-specific genes and proteins include, but are not limited to, MyoD, Myf5, MRF4, myogenin, Pax3, Pax7, desmin, creatine phosphokinase, muscle-specific myosin, Mind bomb 2,  $\alpha$ -actin, tropomyosin-I, actinin, MyHC, zeugmatin, titin, nebulin and MyBP-C. The myoblastic phenotype encompasses the ability of a myoblast to fuse to generate a muscle cell and form myotubes or to de-differentiate into a satellite cell.

Cells of the myogenic lineage include muscular stem cell, myoblast and myoblast-derived cells. Muscular stem cells are known to differentiate into myoblasts. Myoblast-derived cells include, but are not limited to, muscle cells and satellite cells. Muscle cells or muscle fibers have an elongated, cylindrical shape and are multinucleated. The nuclei of these muscles are located in the peripheral aspect of the cell (Totsuka 1987), just under the plasma membrane, which vacates the central part of the muscle fiber for myofibrils. In the heart, the muscle cells are also referred to as myocytes (also known as myocardial cells). Each myocardial cell contain myofibrils, which are long chains of sarcomeres, the contractile units of the cell. Myocytes show similar pattern to skeletal muscle cells, unlike multinucleated skeletal cells, myocytes contain only one nucleus. Satellite cells are small mononuclear progenitor cells with very little cytoplasm and can be found in the mature muscle. They are usually located between the basal lamina and sarcolemma of individual muscle fibers. Satellite cells are able to differentiate and fuse to augment existing muscle fibers and to form new fibers. In undamaged muscle, the majority of satellite cells are quiescent. In response to a mechanical strain, satellite cells become activated; they initially proliferate as skeletal myoblasts before undergoing myogenic differentiation. Satellite cells express a number of distinctive genetic markers such as Pax7 and Pax3. Activated satellite cells also express myogenic transcription factors, such as Myf5 and MyoD. Differentiated satellite cells express muscle-specific filament proteins such as desmin.

The cells that can be cultured in the serum-free medium are preferably those of the myogenic lineage. These cells include, but are not limited to, commercially available cell lines (such



as the C<sub>2</sub>C<sub>12</sub> cell line) as well as primary cultures of cells. In the latter, the cells can be obtained from a tissue biopsy (such as a biopsy of a healthy muscle tissue) or can be derived from stem cells (such as those present in blood (such as cord blood), skin, muscles, etc). Cells of the myogenic lineage of any origin (such as mammalian cells, murine cells and human cells) can be successfully cultured in the serum-free medium. The serum-free medium could be used for the culture of adherent cells and non-adherent cells.

As herein described, the serum-free culture medium allows the proliferation of the cells of the myogenic lineage at a rate similar to the rate that would be obtained with a standard medium with serum. As used herein, the expression "similar rate of proliferation" refers to a rate of proliferation that is either not statistically different or superior to cells cultured in standard serum-containing medium. As shown below in the Examples, commercially available serum-free culture media (such as the SKGM® medium from Lonza) for cells of the myogenic lineage do not enable the growth and proliferation of the cells at a rate similar to the standard medium with serum. The culture medium proposed herein provides a tangible advantage when compared to the existing media in the art.

The culture medium contains at least two components (i) a basal medium and (ii) a cytokine. The basal medium provides a source of consumable energy for the cells, a source of amino acids, minerals, vitamins, salts, trace elements. The basal medium can be either a single medium or a combination of more than one medium. Basal medium contemplated for incorporation into the serum-free medium can be, for example, Dulbecco's Modified Eagle's Medium (DMEM), advanced DMEM, BIOGRO™, SKGM®, Ham's F10, Ham's F12, Iscove's modified Dulbecco's medium, neurobasal medium, RPMI 1640 and/or MCDB120. In a preferred embodiment, the basal medium is a combination of Ham's F12, RPMI 1640 and MCDB120 media. In another embodiment, the ratio between these three basal media is approximately 1:1:1.

The second component of the culture medium is a cytokine. As it is known in the art, cytokines play a crucial role in the activation, growth, proliferation and differentiation of myoblasts. For example, when the muscle is injured, the first phase of muscular repair is necrosis of damaged parts and the activation of the inflammatory response (Charge and Rudnicki 2004). The latter is characterized by the secretion of cytokines by damaged fibers and the cells of the immune system. These cytokines activate quiescent satellite cells who then enter the cellular cycle and proliferate. This activation triggers myogenesis, a series of complex events which involves the differentiation of satellite cells into muscle precursor cells, and the fusion of the later to form muscle fibers. Finally, once the muscle repaired, the inflammatory response fades and the satellite cells return to their quiescent state (Miller et al. 2000; Muntoni et al. 2002).

In order to develop the serum-free culture medium, Applicant has investigated which cytokines could become valuable potential additives to the basal medium. Cytokines are often specific to a cell type, play several important roles in the body, regulate many processes, influence cellular survival, growth and differentiation, etc. In myoblasts, cytokines are also involved in the assembly of the actin cytoskeleton and the mobility of the cells (Kurek et al. 1998; Cooper and Hausman, 2007). Generally, cytokines have the following characteristics:

They have different effects on different cell or tissue, due to the difference in expression of their respective receptors on any given cell type (Schlessinger and Ullrich, 1992; Fitzgerald et al. 2001).

Different cytokines can induce similar reactions since several receptors share similar intracellular signaling pathways. These cytokines are thus known as redundant.

The concentration of cytokines needed to induce an effect is very low, (in the ng/ml range) since receptor affinity for its ligand is generally very high and binding often leads to signal amplification.

Cytokines are part of complex reaction networks, an act in either positive or negative synergy with other cytokines. Multiple cytokines also act in sequence to lead to the complete response (Fitzgerald et al. 2001; Hancock, 2005; Lewin, et al. 2007).

New cytokines are discovered each year, but until now, 80 are known and their mass varies between 10 and 70 kDa (Hancock 2005). Cytokines were originally described as soluble polypeptides used as communication agents between lymphoid cells. However, cytokine definition is now broader and designate any protein which acts on proliferation or differentiation, whereas "growth factor" (GF) is used specifically to define cytokines with a positive effect on cellular division.

Several GF are secreted by macrophages during the immune response and by skeletal muscle cells in an autocrine manner. Generally, they act as progression factors of the cell cycle that prevent the formation of myotubes, but would also contribute to the maturation of muscle fibers and the migration of myoblasts (Kinoshita et al. 1995; Allen et al. 2003; Lafreniere et al. 2004).

Until now, around 50 GF have been identified and several affect muscle cells, such as family members of the fibroblast growth factors (FGFs), epidermal growth factors (EGF, HB-EGF, TGF $\alpha$ ), insulin-like growth factor (IGFs), transforming growth factors (TGFs), bone morphogenic factors (BMPs), hepatocyte growth Factor (HGF), leukemia inhibitory factor (LIF), platelet-derived growth factor (PDGFs), vascular-endothelial growth factor (VEGF), macrophage colony-stimulating factor (M-CSF) and nerve growth factor (NGF) (Floss et al. 1997; Fitzgerald et al. 2001). Some GF receptors are expressed in several types of cells (FGF, EGF, IGF-1) whereas others, such colony-stimulating factor (CSF) and the NGF, are more specific (Schlessinger and Ullrich, 1992).

Interleukins are the principal group of hematopoietic cytokines. They allow communication between cells of the immune and inflammatory systems and control the growth and the differentiation of several cell types. The macrophages and the monocytes produce several types of interleukins that could play an important role in muscle skeletal cell proliferation. To our knowledge, very few project studied the effects of interleukins on muscular cell proliferation, which makes their investigation interesting.

The most studied hematopoietic cytokines are the leukemia inhibitory factor (LIF) of the IL-6 family, IL-1 ( $\alpha$  and  $\beta$ ) and TNF $\alpha$ . LIF specifically stimulates the proliferation and the survival of myoblasts, without supporting those of fibroblasts. TNF $\alpha$  pushes satellite cells to enter and to remain in the cell cycle, induced IL-6 production, stimulates angiogenesis, increases migration and acts as mitogen for rat myoblasts (Austin et al. 1992; White et al. 2001; Ferrara et al. 2003; Li, 2003; Torrente et al. 2003; Langen et al. 2004; Chevrel et al. 2005). Finally, IL-1 is a cytokine which modulates cellular proliferation by the induction and the inhibition of others cytokines and is a precursor of the inflammatory response (Wang et al. 2005). For example, IL-1 $\beta$  induces the

production of IL-6, arachidonic acid, human growth hormone, nitric oxide and certain inflammatory proteins, such as collagenase and elastase, in myoblasts (Mizel, 1989; Fitzgerald et al. 2001; Adams et al. 2002; Chevrel et al. 2005). Moreover, it would prevent IGF-1 from promoting protein synthesis, down-regulating myogenin by the means of ceramide production, a secondary messenger (Broussard et al. 2004; Strle et al. 2004).

In the serum-free medium, many cytokines (such as those listed above) can be added. They can be in a purified form, derived from humans or animals or they can be obtained through recombinant technology. Many cytokines are readily available commercially. In one embodiment, the cytokines can either be one or more growth factors (such as FGF, EGF, BMP, TGF $\beta$  or IGF), and/or one or more interleukins (such as IL-1 to IL-33), and/or one or more hematopoietic cytokines (such as ILs, EPO or M-CSF). The concentration of the growth factors varies but it is usually in a range between about 1 to 100 ng/ml. The concentration of interleukin also varies, but it is usually in a range between about 0.1 to 100 ng/ml. When selecting the cytokine to be added in the serum-free medium, one should take care in selecting a cytokine or a combination of cytokines that it will allow the proliferation of the cells of the myogenic lineage, limit cell death or senescence and enable the cells to retain their ability to differentiate into muscle cells.

The culture medium could also comprise a supplement. This supplement will allow the proliferation of the cells of the myogenic lineage, limit cell death or senescence and/or enable the cells to retain their ability to differentiate into muscle cells. Such supplements can include, but are not limited to (i) a combination of insulin, transferrin and selenite (also known as ITS), (ii) a commercially available B27 supplement (content listed in Table 5, as well as in Brewer et al. 1993), (iii) a combination of dexamethasone, insulin, EGF, fetuin and albumin (also known as Ham's modified additive supplement described in Ham et al. 1988, as well as in Table 1) and/or (iv) a combination of dexamethasone, insulin, bFGF and albumin (Table 2). The concentration of these supplement will vary depending on their intended use. Usually, the concentration of a ITS 100 $\times$  solution ranges between 0.005% and 2.5% (v/v).

Antimicrobial compounds (such as antibiotics) can also be added to the medium to prevent microbial growth during culture, inasmuch as they do not alter cell proliferation, cell death and/or ability to fuse or differentiate.

Lipids can be useful in the culture of cells and can be added to the serum-free medium. Examples of useful lipids includes arachidonic acid, cholesterol, DL- $\alpha$ -tocopherol acetate, linoleic acid, linolenic acid, myristic acid, oleic acid, palmitoleic acid, palmitic acid and stearic acid. The concentration of lipids in the culture medium is usually between 0.05% and 5% (v/v). Lipids added in the serum-free medium should allow the proliferation of the cells of the myogenic lineage, limit cell death or senescence and enable the cells to retain their ability to differentiate into muscle cells.

Surfactants can also be added in the serum-free medium, as long as they allow the proliferation of the cells of the myogenic lineage, limit cell death or senescence and enable the cells to retain their ability to differentiate into muscle cells. Several surfactants can be used, for example pluronic F-68® and TWEEN® (such as TWEEN 80®).

The present application also relates to a method of culturing a cell of the myogenic lineage. In an embodiment, this method comprises the step of contacting the cell with the culture medium described herein. Any cell of the myogenic lineage (such as those described herein) can be used in this

method. The method contemplates the culture of cells on a surface (such as a culture appropriate container or a bead) and the culture of non-attached cells (such as cell lines that form free-floating aggregates in culture). The method can be used for short-term culture period (such as about an hour or more than an hour or about a day or more than one day), mid-term culture period (such as about a week or more than a week) or long-term culture period (such as about a month or more than a month). As described above, the culture medium enables the proliferation of cells at a rate similar of those cultured in a serum-containing medium. Consequently, the method provided herewith does not alter the proliferating rate of cells when compared to traditional methods using serum. The method also has the advantage of limiting cell differentiation in culture while retaining the cell's ability to differentiate when they are placed in a differentiating culture medium or in vivo.

Another advantage of the culture method presented herewith is that it can be used in the scaling up of large volumes of culture (for example, between 1 to 100 liters).

Because the culture medium described herein is free of serum, it can be safely use for the ex vivo expansion of cells that will be implanted or grafted in a patient in need thereof. As described above, the treatment of muscle deficiencies using ex vivo cultured cells is already known in the art. However, because the culture medium described herewith enables the in vitro proliferation of cells of the myogenic lineage while retaining the cultured cell's ability to differentiate (e.g. fuse), the present invention provides a new alternative for providing cells for therapy of a muscular deficiency. It is known in the art that the success of such cellular therapy is linked to the number of cells that can be transferred to the patient. Because the culture medium presented herewith (as well as its corresponding method of using the culture medium) does not contain serum (a risk factor for a potential microbial/prion infection) and enables the proliferation of cells in vitro, it is contemplated that it could be successfully used in cellular therapy.

As such, the present application also relates to the treatment or the alleviation of symptoms of a muscular deficiency in a subject in need thereof. The method comprises contacting a cell of the myogenic lineage with the culture medium described herein or culturing a cell of the myogenic lineage according the method described herein. The cell is then implanted in a subject in need thereof to treat or alleviate the symptoms associated to a muscular deficiency. As used herein, the term "muscular deficiency" refer to a significant reduction in muscle mass in a subject, modifying abilities to maintain posture, movement and ultimately vital functions. Muscular deficiency are, for example, any muscular dystrophy (such as Duchenne, Becker, limb girdle, congenital, facioscapulohumeral, myotonic, oculopharyngeal, distal, and Emery-Dreifuss dystrophy), myocardial infarction and urethral sphincter insufficiency.

The present application also contemplates the use of the serum-free culture medium and cells cultured therein for the treatment or the alleviation of symptoms of a muscular deficiency or dysfunction.

The present invention will be more readily understood by referring to the following examples which are given to illustrate the invention rather than to limit its scope.

#### Example I

##### Methodology Used

Cell culture and media. Myoblasts used in the following examples were derived from three digested human muscular

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biopsies, BB13, H49 and H51. BB13 cells were separated from fibroblasts following the protocol described in Belleslles et al., 1993, and were therefore at least 95% desmin positive (DakoCytomation clone DE-R-11, code M0724) and 95% NKH1 (Beckman-Coulter). H49 and H51 cells were not purified and contained >50% desmin positive-cells. The cells were initially cultured in MCDB120 basal medium (Hyclone SH3A11704.01), complemented with 15% fetal bovine serum (FBS, Hy-Clone SH30396.03, lot KPF21343) and the modified Ham's supplement (refer to Table 2 below). This medium was also used as a positive control, standard (STD) medium. This STD medium is the one presently used to support myoblast proliferation for current clinical trials of in vivo muscular treatments.

TABLE 1

Culture medium supplement proposed by Ham (Ham et al. 1988)	
Additives	Concentration
Dexamethasone	0.39 µg/L
Insulin	0.18 mg/ml
EGF	10 ng/ml
Bovine Albumin	0.5 mg/ml
Fetuin	0.5 mg/ml

TABLE 2

Modified culture medium supplement used in the standard medium	
Additives	Concentration
Dexamethasone	0.39 µg/L
Insulin	5 µg/ml
bFGF	10 ng/ml
Bovine albumin	0.5 mg/ml

TABLE 3

Cytokines and additives mixture added in LOBSFM	
Cytokines//Additives	Concentration
bFGF	4 ng/ml
FGF4	4 ng/ml
EGF	4 ng/ml
IGF1	4 ng/ml
IL1α	4 ng/ml
IL1β	4 ng/ml
BSA	0.5 mg/ml
Fetuin	0.5 mg/ml
Fibronectin	5 µg/ml
Dexamethasone	0.39 µg/ml
ITS	1 X
Lipids	1 X
B27	1 X

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The serum-free medium (also referred to as LOBSFM or LOB-SFM) is composed of a mixture of three basal media. The three basal media are Ham F12 (Invitrogen 11765-054), RPMI1640 (Invitrogen 21870-076) and MCDB120 (Hyclone SH3A1704.01, lot # AQE23666) combined in a 1:1:1 ratio. The compositions of those three basal media are described in Table 4 and Table 5. This mixture is supplemented with six growth factors/cytokines and seven additives (Table 3), Ham's growth supplement cocktail (Table 1) and its modified version (Table 2).

The commercially available medium from Lonza, (SKGM®, Lonza CC-3160) is composed of a basal medium (SKGM®) and an additive mixture (BULLETKIT™) similar in composition to the Ham's supplement (Table 1): insulin (CC-4025N), rhEGF (CC-4017N), BSA (CC-4160N), fetuin (CC-4140N), GA-1000 (CC-4081N) and dexamethasone (CC-4150N).

TABLE 4

Amino acid content of Ham F12, RPMI 1640 and MCDB120 media				
	Ham's F12 (mg/L)	RPMI 1640 (mg/L)	MCDB120 (mg/L)	LOBSFM (mg/L)
Basal media Amino acids (mg/L)				
Glycine	7.5	10	2.25	6.58
L-Alanine	8.9		2.67	3.86
L-Alanine-L-Glutamine	217			72.33
L-Arginine		200		66.67
L-Arginine hydrochloride	211		210.67	140.56
L-Asparagine	13	50		21
L-Asparagine e-H <sub>2</sub> O			15.01	5
L-Aspartic acid	13.3	20	13.31	15.54
L-Cysteine hydrochloride-H <sub>2</sub> O			35.13	11.71
L-Cystine 2HCl	36	65		33.67
L-Glutamic Acid	14.7	20	4.41	13.04
L-Glutamine		300	1461.5	587.17
L-Histidine		15		5
L-Histidine hydrochloride-H <sub>2</sub> O	21		41.93	20.98
L-Isoleucine	4	50	65.58	39.86
L-Leucine	13.1	50	131.17	64.76
L-Lysine hydrochloride	36.5	40	181.65	86.05
L-Methionine	4.5	15	29.84	16.45
L-Phenylalanine	5	15	33.04	17.68
L-Proline	34.5	20	11.51	22
L-Serine	10.5	30	31.53	24.01
L-Threonine	11.9	20	35.73	22.54
L-Tryptophan	2.04	5	4.08	3.71
L-Tyrosine	5.4		18.12	7.84
L-Tyrosine disodium salt dehydrate		29		9.67
L-Valine	11.7	20	117.15	49.62

TABLE 5

Vitamins, salts, trace elements, lipids and other additives contained in Ham F12, RPMI 1640 and MCDB120				
	Ham's F12 (mg/L)	RPMI 1640 (mg/L)	MCDB120 (mg/L)	LOBSFM (mg/L)
Basal media				
Vitamins				
Biotin	0.0073	0.02	0.00733	0.012
Folic Acid	1.3	1		0.767

TABLE 5-continued

Vitamins, salts, trace elements, lipids and other additives contained in Ham F12, RPMI 1640 and MCDB120				
	Ham's F12 (mg/L)	RPMI 1640 (mg/L)	MCDB120 (mg/L)	LOBSEFM (mg/L)
Basal media				
Folinic acid (5-formyl tetrahydrofolate-5H <sub>2</sub> O) (Ca salt)			0.602	0.201
Niacinamide	0.036	1	6.11	2.382
D-Pantotheique acid (Hemi-Ca salt)			23.82	7.94
D-Calcium pantothenate	0.5	0.25		0.25
Pyridoxine hydrochloride	0.06	1	2.056	1.039
Riboflavin	0.037	0.2	0.003764	0.08
Thiamine hydrochloride	0.3	1	3.373	1.558
Cobalamine	1.4	0.005	0.01355	0.473
Choline chloride	14	3	13.96	10.32
i-Inositol	18	35	18.016	23.672
Para-Aminobenzoic Acid		1		0.333
Salts				
CaCl <sub>2</sub> —2H <sub>2</sub> O	44		235.23	93.08
Ca(NO <sub>3</sub> ) <sub>2</sub> —4H <sub>2</sub> O		100		33.33
MgCl <sub>2</sub> —6H <sub>2</sub> O	122			40.67
MgSO <sub>4</sub> —7H <sub>2</sub> O			246.38	82.13
MgSO <sub>4</sub> (anhyd)		48.8		16.28
KCl	223.6	400	298.2	307.27
NaHCO <sub>3</sub>	1176	2000	1176	1450.67
NaCl	7599	6000	6430	6676.33
Na <sub>2</sub> HPO <sub>4</sub> (anhyd)	142	800		314
Na <sub>2</sub> HPO <sub>4</sub> —7H <sub>2</sub> O			134.04	44.68
Traces elements				
(NH <sub>4</sub> ) <sub>6</sub> Mo <sub>7</sub> O <sub>24</sub> —4H <sub>2</sub> O			0.0037	0.0012
HNH <sub>4</sub> VO <sub>3</sub>			0.0006	0.0002
CuSO <sub>4</sub> —5H <sub>2</sub> O	0.003		0.0025	0.0017
FeSO <sub>4</sub> —7H <sub>2</sub> O	0.834		0.834	0.556
MnSO <sub>4</sub> —5H <sub>2</sub> O			0.00024	0.00008
NiCl <sub>2</sub> —6H <sub>2</sub> O			0.00007	0.00002
H <sub>2</sub> SeO <sub>3</sub>			0.00387	0.00129
Na <sub>2</sub> SiO <sub>3</sub> —9H <sub>2</sub> O			2.842	0.947
ZnSO <sub>4</sub> —7H <sub>2</sub> O			0.086	0.029
Lipids				
Linoleic acid	0.1			0.028
Lipoic acid	0.2			0.067
Others				
Adenine			0.135	0.045
D-Glucose	1802	2000	1000	1601
Glutathione (reduced)		1		0.33
Hypoxanthine	4			1.33333
Phenol Red	1.2	5	1.242	2.481
Putrescine 2HCl	0.2		0.00016	0.05372
Sodium pyruvate	110		110.04	73.35
Thymidine	0.7		0.024	0.241

#### Proliferation Assays and Designs of Experiments (DOE).

A frozen aliquot of 1-3×10<sup>6</sup> myoblasts was thawed and diluted to 2×10<sup>5</sup> cells/ml in STD medium in a 75 cm<sup>2</sup> tissue culture T-flask and incubated at 37° C. in a humidified, 5% CO<sub>2</sub> atmosphere. After approximately five days, the culture reached 80% confluence, at which point the cells were trypsinized, rinsed and centrifuged three times with phosphate buffer saline (PBS), and inoculated in cell culture multiwell plates (either 6 or 24 wells) at 10 000 cells/ml in the different culture media. No adaptation of cells for serum-free conditions was made before the experiments. Media change was performed every two to three days and cells were counted using a hemacytometer.

#### Immunostaining Assays.

Cells recuperated from an enzymatic digestion of a muscular biopsy form a heterogeneous cellular population, even

when myoblast enrichment is performed. An efficient myoblast culture medium will not only allow extensive and rapid proliferation of any cells, but will also specifically favor myoblast proliferation. Consequently, characterization of cell population at different points in time during their expansion is important. To do so, immunostaining was performed, and the degree of expression of myoblast specific markers was assessed either by microscopy or flow cytometry. The markers used in this study were desmin (DakoCytomation clone DE-R-11, code M0724), NKH-1 (NKH1-1-RD1/NCAM/CD56; Beckman Coulter 6603067) and myosin heavy chain (MHC; MF20 (Iowa Hybridoma Bank)). Desmin is a highly specific marker for activated satellite cells and myogenic precursors. The anti-desmin antibody stains the intermediate filaments of the cytoskeleton (Freshney 2005). NCAM (neural cell adhesion molecule) is a glycoprotein found at high

concentration on the plasma membrane of myoblasts and progenitors of muscular skeletal cells (Nomura, Ashihara et al. 2007), but also on neurons and on natural killer cells (NK). NCAM is thus a little less specific than desmin for myoblasts. MHC indicates the formation of myotubes. DAPI (Sigma D8417) was also used to color the nucleus. In order to perform desmin and MHC staining, cells were rinsed and agitated three times with PBS, incubated 5 minutes each time with fresh PBS; cells were fixed with a solution of ethanol 95%, incubated 10 minutes, and discarded; a solution of PBS supplemented with 10% FBS was added, let to incubate one hour, and discarded; a solution of PBS supplemented with 10% FBS and the antibody (anti-desmin or anti MHC) was added, incubated one hour, and discarded; cells were rinsed and agitated three times in PBS, 5 minutes each time with fresh PBS; a solution of PBS supplemented with 10% FBS and the labeled secondary antibody (with ALEXAFLUOR 488™ or ALEXAFLUOR 546™) was added, incubated one hour, and discarded; cells were rinsed and agitated three times in PBS, 5 minutes each time with fresh PBS; and DAPI was added on the last rinse. At least 500 cells are counted for each condition to allow for proper result accuracy.

## Fusion Assay.

Culture medium was removed from the culture well when 80%-90% confluence was reached and it was replaced by a differentiation medium. The differentiation medium was composed of RPMI1640/F12/MB1 completed only with ITS (10 µg/ml insulin, 5.5 µg/ml transferrin, 6.7 ng/ml sodium selenite). Under these conditions, normal myoblasts usually differentiate and fuse to form myotubes within three to four days.

Reverse-Transcriptase Polymerase-Chain-Reaction (RT-PCR).

RT-PCR was used to probe the transcriptome of receptors and autocrine factors expressed by myoblasts, as well as the growth factors expressed by macrophage, since it has been reported that macrophage conditioned medium promotes myoblast proliferation (Cantini et al. 1994; Massimino et al. 1997; Caroleo et al. 2001; Cantini et al. 2002; Chazaud et al. 2003; Tidball, 2005). BB13 cells were used for myoblast expression profiling, since they are a 95-100% pure myoblast population. A monocyte/macrophage cell line (ATCC CRL-9855) was also used to study macrophage expression. The ligands of myoblast receptors were also surveyed. Primer for each of these genes were found in the scientific literature and synthesized.

TABLE 6

Primers sequences for myoblast receptors, ligands and muscle-specific genes.			
Target mRNA (NCBI Ref. No.)	Forward	Reverse	Size (bp) Temp. (° C.)
β-actin (NM_001101.2)	AGCCTCGCCTTTGCCGA (SEQ ID NO: 1)	CTGGTGCCTGGGGCG (SEQ ID NO: 2)	175 (54)
activin-RIIA (NM_001616.3)	TACTGCTGCAGATGGACCTG (SEQ ID NO: 3)	AGCTCCAGTTCAGAGTCCC (SEQ ID NO: 4)	389 (60)
BMP-R1α (NM_004329.2)	GCAATTGCTCATCGAGACC (SEQ ID NO: 5)	CGAAGGTGTAGATGTCAGCC (SEQ ID NO: 6)	232 (58)
BMP-R1β (NM_001203.1)	GCAGCACAGACGGATATTGT (SEQ ID NO: 7)	TTTCATGCCTCATCAACACT (SEQ ID NO: 8)	630 (56)
BMP-R2 (NM_001204.5)	ACGGGAGAGAAGACGAGCCT (SEQ ID NO: 9)	CTAGATCAAGAGAGGGTTCG (SEQ ID NO: 10)	694 (60)
C-MET (NM_000245.2)	GGTCAATTCAGCGAAGTCCT (SEQ ID NO: 11)	TTCTGTATCTTCTCCAGTG (SEQ ID NO: 12)	242 (56)
COL1 (NM_000088.3)	GGAACAGACAAGCAACCCAACT (SEQ ID NO: 13)	GGTCATGTTTCGGTTGGTCAAAGATAA (SEQ ID NO: 14)	142 (55)
Desmin (NM_001927.3)	TCTGAAGCTGAGGAGTGGTA (SEQ ID NO: 15)	CTTCTTGGTATGGACCTCAG (SEQ ID NO: 16)	465 (60)
DHH (NM_021044.2)	GTTGTAAGGAGCGGGTGAAC (SEQ ID NO: 17)	GCCAGCAACCCATACTTGTT (SEQ ID NO: 18)	184 (58)
EGF (NM_001963.2)	GGTCAATGCAACCAACTTCA (SEQ ID NO: 19)	GGCATTGAGTAGGTGATTAG (SEQ ID NO: 20)	383 (52)
EGF-R (NM_005228.3)	ATGTCCGGGAACACAAAGAC (SEQ ID NO: 21)	TTCCGTCATATGGCTTGAT (SEQ ID NO: 22)	351 (58)
EPO (NM_000799.2)	GATAAAGCCGTCAGTGGCCTTC (SEQ ID NO: 23)	GGGAGATGGCTTCTTCTGGG (SEQ ID NO: 24)	76 (60)
EPOR (NM_000121.2)	CCTGACGCTCTCCCTCATCC (SEQ ID NO: 25)	GCCTTCAAACCTCGCTCTCTGG (SEQ ID NO: 26)	130 (60)

TABLE 6-continued

Primers sequences for myoblast receptors, ligands and muscle-specific genes.			
Target mRNA (NCBI Ref. No.)	Forward	Reverse	Size (bp) Temp. (° C.)
FGF-1 (NM_033136.1, NM_033137.1, NM_000800.2)	CATGGCTGAAGGGGAAATCAC (SEQ ID NO: 27)	AATCAGAAGAGACTGGCAGGGG (SEQ ID NO: 28)	363 (64)
FGF-2 (NM_002006.4)	TCACCAGCTGCCCGCCTTGC (SEQ ID NO: 29)	CAGTTCGTTTCAGTGCCACAT (SEQ ID NO: 30)	375 (62)
FGF-3 (NM_005247.2)	TTGGAGATAACGGCAGTGGA (SEQ ID NO: 31)	CTCCAGGTTATCCGGGCTCT (SEQ ID NO: 32)	438 (60)
FGF-5 (NM_004464.3, NM_033143.2)	GCTGTGTCTCAGGGGATTGTAGGAATA (SEQ ID NO: 33)	TATCCAAAGCGAAACTTGAGTCTGTA (SEQ ID NO: 34)	434 (65)
FGF-7 (NM_002009.2)	ACCCGGAGCACTACACTATAATGC (SEQ ID NO: 35)	TTATTGCCATAGGAAGAAAGTGGG (SEQ ID NO: 36)	600 (68)
FGF-10 (NM_004465.1)	GAGATGTCCGCTGGAGAAAG (SEQ ID NO: 37)	ATTGCTCTCCATTATGCTG (SEQ ID NO: 38)	304 (60)
FGFR-1 (NM_023110.2, NM_015850.3, NM_023105.2, NM_023106.2, NM_023107.2, NM_023111.2)	AAGGTCCGTTATGCCACCT (SEQ ID NO: 39)	CAGGTTGTCTGGGCCAATC (SEQ ID NO: 40)	297 (58)
FGFR-2 (NM_022970.2, NM_000141.3)	GGAAAAGAACGGCAGTAAAT (SEQ ID NO: 41)	GTAGTCTGGGAAGCTGTAA (SEQ ID NO: 42)	247 (56)
FGFR-3 (NM_000142.2, NM_022965.1)	GGGCCCCCTTACTGGACACG (SEQ ID NO: 43)	GCCGGATGCTGCCAAACT (SEQ ID NO: 44)	270 (58)
FGFR-4 (NM_002011.3, NM_022963.2, NM_213647.1)	CGCTGGCTTAAGGATGGA (SEQ ID NO: 45)	CTGCCCACAGCGTTCTCT (SEQ ID NO: 46)	149 (56)
FLRG- follistatin- related gene (NM_005860.2)	ACCTGAGCGTCATGTACCG (SEQ ID NO: 47)	TGTGGCACGAGGAGATGTAG (SEQ ID NO: 48)	198 (60)
GAPDH (NM_002046.3)	GCCAAGGTCATCCATGACAAC (SEQ ID NO: 49)	GTCCACCACCCTGTTGCTGTA (SEQ ID NO: 50)	498 (60)
G-CSF (NM_172220.1, NM_172219.1, NM_000759.2)	AGCTTCCTGCTCAAGTGCTTAGAG (SEQ ID NO: 51)	TTCTTCCATCTGCTGCCAGATGGT (SEQ ID NO: 52)	336 (72)
GM-CSF (NM_000758.2)	GTCTCCTGAACCTGAGTAGAGACA (SEQ ID NO: 53)	AAGGGGATGACAAGCAGAAAGTCC (SEQ ID NO: 54)	286 (722)
GM-CSFR $\alpha$ (NM_172249.1, NM_172247.1, NM_172245.1, NM_006140.3, NM_172246.1)	CTTCTCTCTGACCAGCA (SEQ ID NO: 55)	ACATGGGTTCTCTGAGTC (SEQ ID NO: 56)	546 (60)
GM-CSFR $\beta$ (NM_000395.1)	TGGAGTGGCCTCTGGTTATG (SEQ ID NO: 57)	GGGAAGTAGGGAGACAGACGAG (SEQ ID NO: 58)	82 (62)
HB-EGF (NM_001945.1)	GGTGTGAAGCTCTTTCTGGCTGC (SEQ ID NO: 59)	ATTATGGGAGGCCCAATCCTAGAC (SEQ ID NO: 60)	754 (72)

TABLE 6-continued

Primers sequences for myoblast receptors, ligands and muscle-specific genes.			
Target mRNA (NCBI Ref. No.)	Forward	Reverse	Size (bp) Temp. (° C.)
HGF (NM_000601.4, NM_001010932.1)	CTACACTGGATTGATCAACTAT (SEQ ID NO: 61)	AGTAGTTGTCTTAGGATTGTTG (SEQ ID NO: 62)	443 (52)
IGF-1 (NM_000618.2)	AAATCAGCAGTCTTCCAACC (SEQ ID NO: 63)	CTTCTGGGTCTTGGGCATGT (SEQ ID NO: 64)	395 (57)
IGF-1R (NM_000875.3)	CTGCTGATGTGTACGTTCCCT (SEQ ID NO: 65)	TCAGGTTTCATCTTTCACCAC (SEQ ID NO: 66)	133 (56)
IGF-II (NR_003512.1, NM_000612.3, NM_001007139.3)	CCTGGAGACGTACTGTGCTA (SEQ ID NO: 67)	GGACTGCTTCCAGGTGTC (SEQ ID NO: 68)	132 (56)
IGF-IIR (NM_000876.2)	GCTGTTTGTATGCACGACTT (SEQ ID NO: 69)	TGCTCTGGACTCTGTGATTT (SEQ ID NO: 70)	142 (56)
IHH (NM_002181.2)	CTACGCCCCGCTCACAAAG (SEQ ID NO: 71)	GGCAGAGGAGATGGCAGGAG (SEQ ID NO: 72)	376 (60)
IL-1 $\alpha$ (NM_000575.3)	GTCTCTGAATCAGAAATCCTTCTATC (SEQ ID NO: 73)	CATGTCAAATTTCACTGCTTCATCC (SEQ ID NO: 74)	421 (58)
IL-1 $\beta$ (NM_000576.2)	TGAACTGAAAGCTCTCCACC (SEQ ID NO: 75)	CTGATGTACCAGTTGGGGAA (SEQ ID NO: 76)	297 (60)
IL-2 $\gamma$ (NM_000206.1)	GGAAGCCGTGGTTATCTCTGTT (SEQ ID NO: 77)	GGTGGGTTGAATGAAGGAAAGT (SEQ ID NO: 78)	407 (58)
IL-2R $\alpha$ (NM_000417.1)	ATCCACACGCCACATTCAAAGC (SEQ ID NO: 79)	TGCCCCACCACGAAATGATAAAT (SEQ ID NO: 80)	347 (58)
IL-2R $\beta$ (NM_000878.2)	GCCCCATCTCCCTCCAAGT (SEQ ID NO: 81)	AGGGGAAGGGCGAAGAGAGC (SEQ ID NO: 82)	529 (66)
IL-3R $\alpha$ (NM_002183.2)	ACCCACCAATCACGAACCTAAG (SEQ ID NO: 83)	GGTCACATTTCTGTAAAGGTCCC (SEQ ID NO: 84)	74 (66)
IL-4 (NM_000589.2, NM_172348.1)	ACTCTGTGCACCGAGTTGACCGTAA (SEQ ID NO: 85)	TCTCATGATCGTCTTTAGCCTTTCC (SEQ ID NO: 86)	300 (55)
IL-4R $\alpha$ (NM_001008699.1, NM_000418.2)	TGCGTCTCCGACTACATGAG (SEQ ID NO: 87)	TGACTGCATAGGTGAGATG (SEQ ID NO: 88)	385 (60)
IL-5 (NM_000879.2)	TGCCTACGTGTATGCCATCCC (SEQ ID NO: 89)	CTTGGCCCTCATTCTCACTGC (SEQ ID NO: 90)	438 (60)
IL-5R (NM_000564.2, NM_175725.1, NM_175724.1, NM_175726.1, NM_175727.1, NM_175728.1)	CCCTGAGGACACGAGTATT (SEQ ID NO: 91)	TGATCAAAGGGCCTGATAGC (SEQ ID NO: 92)	198 (60)
IL-6 (NM_000600.2)	ATGAACCTCTCTCCACAAGCGC (SEQ ID NO: 93)	GAAGAGCCCTCAGGCTGGACTG (SEQ ID NO: 94)	628 (58)
IL-6R (NM_000565.2)	CATTGCCATTGTTCTGAGGTTT (SEQ ID NO: 95)	AGTAGTCTGTATTGCTGATGTC (SEQ ID NO: 96)	251 (60)
IL-7 (NM_000880.2)	TCTAATGGTCAGCATCGATCA (SEQ ID NO: 97)	GTGGAGATCAAAATCACCAG (SEQ ID NO: 98)	190 (60)
IL-7R (NM_002185.2)	TGCTCAAAATGGAGACTTGG (SEQ ID NO: 99)	GAGGGCCCCACATATTTCA (SEQ ID NO: 100)	160 (60)
IL-10 (NM_000572.2)	TGAGAACCAAGACCCAGACA (SEQ ID NO: 101)	TCATGGCTTTGTAGATGCCT (SEQ ID NO: 102)	182 (60)

TABLE 6-continued

Primers sequences for myoblast receptors, ligands and muscle-specific genes.			
Target mRNA (NCBI Ref. No.)	Forward	Reverse	Size (bp) Temp. (° C.)
IL-11 (NM_000641.2)	ATGAACGTGTGTTTGCCGCTG (SEQ ID NO: 103)	GAGCTGTAGAGCTCCCAGTGC (SEQ ID NO: 104)	270 (64)
IL-12 (NM_002187.2)	TCACAAAGGAGGCGAGGTTTC (SEQ ID NO: 105)	TGAACGGCATCCACCATGAC (SEQ ID NO: 106)	378 (62)
IL-13R $\alpha$ 1 (NM_001560.2)	AAGGAATACCAGTCCCGACA (SEQ ID NO: 107)	ACCAGGGAACCATGAAACAAG (SEQ ID NO: 108)	457 (60)
IL-13R $\alpha$ 2 (NM_000640.2)	GTGAAACATGGAAGACCATC (SEQ ID NO: 109)	GTGAAATAACTGGATCTGATAGGC (SEQ ID NO: 110)	454 (60)
IL-15 (NM_172174.1 NM_000585.2)	TCTTCATTTTGGGCTGTTTCA (SEQ ID NO: 111)	GTGAACATCACTTCCGTATA (SEQ ID NO: 112)	143 (60)
IL-15R $\alpha$ (NM_002189.2 NM_172200.1)	CGCCAGGTGTGTATCCAC (SEQ ID NO: 113)	GTTTGCCTTGACTTGAGGTA (SEQ ID NO: 114)	121 (60)
IL-17 (NM_002190.2)	TCCCCAGTTGATTGGAAGA (SEQ ID NO: 115)	AGTCAAACCTTCCTTCTTGGA (SEQ ID NO: 116)	709 (56)
IL-17R (NM_014339.4)	GCTTCACCCTGTGGAACGAATC (SEQ ID NO: 117)	GGAGATGCCCGTGATGAACCA (SEQ ID NO: 118)	329 (66)
IL-18 (NM_001562.2)	ACCTGGAATCAGATTACTTTG (SEQ ID NO: 119)	CCATACCTCTAGGCTGGCT (SEQ ID NO: 120)	195 (60)
IL-20R $\beta$ (NM_144717.2)	CCTTCAGCCAGACAGAATGTGT (SEQ ID NO: 121)	GCAAACAGGGCCAGTACCA (SEQ ID NO: 122)	67 (60)
IL-21R (NM_021798.2 NM_181078.1 NM_181079.1)	TGTGGAGGCTATGGAAGAAGATATG (SEQ ID NO: 123)	GTGCACCCACCCATTTCTTG (SEQ ID NO: 124)	105 (72)
IL-22 (NM_020525.4)	ACAACACAGACGTTCTGTCATTG (SEQ ID NO: 125)	GAACAGCACTTCTTCAAGGGTGA (SEQ ID NO: 126)	113 (58)
IL-22R1 (NM_021258.2)	CCTGAGCTACAGATATGTCACCAAG (SEQ ID NO: 127)	GGCTGGAAAGTCAGGACTCG (SEQ ID NO: 128)	78 (58)
LIF (NM_002309.2)	GCCATACGCCACCCATGTCACAAC (SEQ ID NO: 129)	GTTGGGGCCACATAGCTTGCCAG (SEQ ID NO: 130)	153 (76)
LIFR (NM_002310.3)	GTGGCAGTGGCTGTCATTGTT GGAGTGGT (SEQ ID NO: 131)	TCATCTGCGGCTGGGTTTG GTATTTCTTC (SEQ ID NO: 132)	365 (86)
M-CSF (NM_000757.3 NM_172210.1 NM_172211.1 NM_172212.1)	TTGGGAGTGGACACCTGCAGTCT (SEQ ID NO: 133)	CCTTGGTGAAGCAGCTCTTCAGCC (SEQ ID NO: 134)	249 (72)
MRF4 (NM_002469.1)	GCTCGTGATAACGGCTAAGGAA (SEQ ID NO: 135)	CGATGGAAGAAAGGCATCGA (SEQ ID NO: 136)	80 (60)
Myf5 (NM_005593.2)	ATGGACGTGATGGATGGCTGCCAGTT (SEQ ID NO: 137)	GCGGCACAACTCGTCCCCAAATT (SEQ ID NO: 138)	103 (60)
MyoD (NM_002478.4)	AGCACTACAGCGGCGACT (SEQ ID NO: 139)	GCGACTCAGAAGGCACGTC (SEQ ID NO: 140)	264 (60)
Myogenin (NM_002479.4)	GACATCCCCCTACTTCTACC (SEQ ID NO: 141)	TCACGCTCCTCTGGTTG (SEQ ID NO: 142)	420 (58)
Myosin-HC (NM_005963.3)	TGTGAATGCCAAATGTGCTT (SEQ ID NO: 143)	GTGGAGCTGGGTATCCTTGA (SEQ ID NO: 144)	751 (60)



TABLE 6-continued

Primers sequences for myoblast receptors, ligands and muscle-specific genes.			
Target mRNA (NCBI Ref. No.)	Forward	Reverse	Size (bp) Temp. (° C.)
Myostatin (NM_005259.2)	GGAAACAATCATTACCATGC (SEQ ID NO: 145)	ATCCATAGTTGGGCCTTTAC (SEQ ID NO: 146)	129 (56)
NGF (NM_002506.2)	AGCTTTCTATCCTGGCCACA (SEQ ID NO: 147)	GATCCTGAGTGTCTGCAGCTT (SEQ ID NO: 148)	344 (59)
p75 (NGF receptor) (NM_002507.1)	CCAGAGGGAGAAAACTCCA (SEQ ID NO: 149)	CTGCACAGACTCTCCACGAG (SEQ ID NO: 150)	371 (59)
PAX7 (NM_002584.1 NM_013945.1)	CAAGATTCTTTGCCGCTACC (SEQ ID NO: 151)	TTCAGTGGGAGGTCAGGTTC (SEQ ID NO: 152)	390 (55)
PDGF-A (NM_033023.3 NM_002607.4)	CCCCTGCCCATTCGGAGGAAGAG (SEQ ID NO: 153)	TTGCCACCTTGACGCTGCGGTG (SEQ ID NO: 154)	227 (62)
PDGF-B (NM_002608.1 NM_033016.1)	GATCCGCTCCTTTGATGATC (SEQ ID NO: 155)	GTCTCACACTTGCATGCCAG (SEQ ID NO: 156)	435 (60)
PDGFR $\alpha$ (NM_006206.3)	ATCAATCAGCCCAGATGGAC (SEQ ID NO: 157)	TTCACGGGCAGAAAGGTACT (SEQ ID NO: 158)	891 (60)
PDGFR $\beta$ (NM_002609.3)	AATGTCTCCAGCACCTTCGT (SEQ ID NO: 159)	AGCGGATGTGGTAAGGCATA (SEQ ID NO: 160)	688 (58)
PTC2-Hh receptor (NM_003738.3)	CTGGCTTCGTGCTTACTTCC (SEQ ID NO: 161)	CGGGTGTGAGGATGTTCTCT (SEQ ID NO: 162)	287 (58)
SCF (NM_003994.4 NM_000899.3)	CTCCTATTTAATCCTCTCGTC (SEQ ID NO: 163)	TACTACCATCTCGTTATCCA (SEQ ID NO: 164)	177 (60)
sEPOR (NM_000121.2)	TGATTGGCTCAGTTCACCAG (SEQ ID NO: 165)	AGGTTGCTCAGCACACACTC (SEQ ID NO: 166)	128 (60)
SSH (NM_000193.2)	CGGAGCGAGGAAGGAAAG (SEQ ID NO: 167)	TTGGGGATAAACTGCTTGTAGGC (SEQ ID NO: 168)	262 (58)
TGF $\beta$ -1 (NM_000660.3)	ACCAACTATTGCTTCAGCTC (SEQ ID NO: 169)	TTATGCTGGTTGTACAGG (SEQ ID NO: 170)	197 (55)
TGF $\beta$ -R1 (NM_004612.2)	TCGTCTGCATCTCACTCAT (SEQ ID NO: 171)	GATAAATCTCTGCCTCAG (SEQ ID NO: 172)	342 (54)
TGF $\beta$ -2 (NM_003238.1)	CTGTCCCTGCTGCACTTTTGT (SEQ ID NO: 173)	TCTCCCGCGGTTGGTCTGTT (SEQ ID NO: 174)	227 (58)
TGF $\beta$ -3 (NM_003239.1)	CCTTTCAGCCCAATGGAGAT (SEQ ID NO: 175)	ACACAGCAGTTCTCCTCCAA (SEQ ID NO: 176)	259 (57)
TGF $\beta$ -R2 (NM_003242.5 NM_001024847.2)	GCGGGAGCACCCCTGTGTC (SEQ ID NO: 177)	CCCGAGAGCCTGTCCAGATGC (SEQ ID NO: 178)	213 (62)
TGF $\beta$ -R3 (NM_003243.2)	AATCTGGGCCATGATGCAG (SEQ ID NO: 179)	ACTGCTGTTTTCCGAGGCT (SEQ ID NO: 180)	286 (57)
TNF (NM_000594.2)	TCAGCCTCTTCTCCTTCTCTG (SEQ ID NO: 181)	TGAAGAGGACCTGGGAGTAG (SEQ ID NO: 182)	324 (60)
TNFR-1 (NM_001065.2)	ACCAAGTGCCACAAGGAAC (SEQ ID NO: 183)	CTGCAATTGAAGCACTGGAA (SEQ ID NO: 184)	263 (55)
TNFR-2 (NM_001066.2)	TTCTGCTCTTCCAGTTGGACT (SEQ ID NO: 185)	CACCAGGGGAAGAATCTGAG (SEQ ID NO: 186)	349 (55)

TABLE 6-continued

Primers sequences for myoblast receptors, ligands and muscle-specific genes.			
Target mRNA (NCBI Ref. No.)	Forward	Reverse	Size (bp) Temp. (° C.)
TrkA-NGF receptor (NM_001012331.1 NM_002529.3 NM_001007792.1)	CAATGTCCACGAGTGACCTCAA (SEQ ID NO: 187)	TGAACTCGAAAGGGTTGTCC (SEQ ID NO: 188)	401 (59)

Cellular RNA was 1) purified with TRIZOL®(Gibco 15596), 2) reverse transcribed and 3) amplified. 1) RNA isolation was done according to the manufacturer instructions (GIBCO). Briefly, 1E6 cells were mixed with 1 ml of TRI-ZOL® and incubate at room temperature for 5 minutes. Then, 200 µl/ml TRI-ZOL® of chloroform was added and mixed vigorously for 15 seconds and incubated 3 minutes at room temperature. The mixture was centrifuged for 15 minutes at 12 000 g. The aqueous phase containing RNA was recuperated and 500 µl/ml of TRI-ZOL™ of isopropanol for 15 seconds was added at room temperature to allow the precipitation of RNA. The mixture was incubated 10 minutes at room temperature and centrifuged at 12 000 g for 10 minutes. The supernatant was removed and rinsed with 1 ml/ml TRI-ZOL® of a 75% ethanol/25% distilled H<sub>2</sub>O solution, agitated on vortex few seconds, and centrifuged 5 minutes at 7 500 g. Then, briefly dry RNA samples were solubilized in distilled H<sub>2</sub>O (~40 µl). Total RNA was quantified and assayed for purity with a spectrophotometer (Beckman Coulter DU 650), where a minimum ratio A<sub>260/280</sub>>1.6 was considered valuable (samples were diluted 1/200 in TRIS-EDTA). 2) For reverse transcription (RT), a mixture of Oligo dt (2 µL at 10 µM), 5 mM of each dNTPs (2 µL), RNA (1 µg) and RNase free water was use to complete volume to 17 µl. The mixture was heated to 65° C. for RNA denaturation and cooled on ice. 2 µl of 10× buffer and 1 µl de RT were added. Thermocycling of the samples at 42° C. for 60 minutes and at 70° C. for 15 minutes to inactivate the enzyme complete the RT phase. 3) The mixture for PCR reaction consisted of dNTPs (0.5 µl 25 mM), Taq polymerase (20 U/ml), Taq buffer 10× (5 µl), cDNA (2 µl), forward+reverse primers (2 µl each) and deionized, RNase free water (complete to 50 µl). The cycling conditions were as follow: 1 cycle at 94° C. for 2 minutes; 35 cycles at 94° C. for 30 seconds, annealing temperature for 30 seconds and 72° C. for 45 seconds; 1 cycle at 72° C. for 10 minutes; 1 cycle at 4° C. for an indefinite period. Finally, the amplicons were run on agarose gels to evaluate their concentration and their approximate molecular weight.

#### Statistics.

Calculations were performed using Matlab software and a p-value of 0.05 was deemed sufficient to recognize a significant effect.

#### Example II

##### Comparison of Seven Different Culture Media

The capacity of seven different culture media to support myoblast expansion was compared with second passage (P2) H49 cells. The cells were cultured as described in Example I and were inoculated in 24-well plasma treated plates (Sarstedt 83.1836, 500 µL/well). Cells were cultured in either

15 i) STD medium, or ii) a first generation serum-free medium developed in our lab (MBa, composition described in Table 7) without any other additives, or additioned with either iii) a macrophage conditioned medium (obtained from the supernatant of a macrophage culture (ATCC CRL-9855) in DMEM supplemented with ITS, 48 hours following a stimulation with LPS), iv) IL-1α, v) IL-1β, vi) calpeptine or vii) with a combination of B27 (refer to Table 8 below), N2 and sonic hedgehog (ShH). As shown in FIG. 1, at culture day nine, four additives (MCM, IL-1α, IL-1β, or a combination of B27, N2 and ShH) significantly improved the proliferation of myoblasts compared to MBa alone. However, calpeptine appeared to act as a proliferation/expansion inhibitor.

TABLE 7

Composition of MBa medium		
Additives	Concentration	units
BSA	0.5	mg/ml
Fetuin	0.5	mg/ml
ITS	1X	
Lipid	1X	
Dexamethasone	0.39	µg/ml
bFGF	5	ng/ml
EGF	5	ng/ml
IGF-1	20	ng/ml
PDGF-BB	5	ng/ml
FGF-4	10	ng/ml
RPMI 1640	1/3	—
F12	1/3	—
MCDB 120	1/3	—

TABLE 8

Composition of B27 (Brewer et al. 1993)	
Biotin	Selenium
L-carnitine	T3 (triiodo-L-thyronine)
Corticosterone	DL-a-tocopherol (vitamin E)
Ethanolamine	DL-a-tocopherol acetate
D(+)-galactose	Bovine albumin
Glutathione (reduced)	Catalase
Linoleic acid	Insulin
Linolenic acid	Superoxide dismutase
Progesterone	Transferrin
Putrescin	Retinyl acetate

#### Example III

##### Comparison of Cell Expansion in Standard and Serum-Free Media

LOBSFM was compared to a serum-free medium commercially available from Lonza (SKGM® +BULLETKIT®) and

with the standard medium (STD). BB13 cells in P<sub>4</sub> were cultured as described in Example I. The cells were inoculated in 24-well plasma treated plates (Sarstedt 83.1836, 500  $\mu$ L/well) and cultured in either standard medium, SKGM® serum-free medium completed with the BULLETKIT™ additives, LOBSFM serum-free medium or SKGM® serum-free medium completed with LOBSFM additives. The cell were counted with a hemacytometer at three and five days post-plating. As shown in FIG. 2, cell expansions in the standard medium or in the LOBSFM serum-free medium are similar. However, the expansion of cells in the completed SKGM® serum-free medium or in the SKGM® medium with LOBSFM additives is less important than in the standard medium or in the LOBSFM medium.

#### Example IV

##### Comparison of Long Term Expansion of Cells in Standard and Serum-Free Media

The reproducibility of long term myoblast proliferation in the LOBSFM and the STD medium was also determined. BB13 cells in P<sub>4</sub> were cultured as described in Example I. The cells were trypsinized and inoculated in 24-well plasma treated plates (Sarstedt 83.1836, 500  $\mu$ L/well) at a concentration of 10 000 cells/ml. The results shown in FIG. 3 represent two experiments carried out independently. The monitoring of cellular expansion accounts for the subsequent dilution of the culture, and is therefore expressed in expansion fold. As shown in FIG. 3, there is no statistical difference between cell expansions in STD medium or LOBSFM medium.

#### Example V

##### Comparison of Myoblast Specific Antigen and Gene Expressions for Cells Cultured in Standard and Serum-Free Medium

In order to verify that the LOBSFM medium allowed the specific expansion of myoblasts, desmin and NKH-expression was imaged using antibodies. The expression of myoblast-specific antigen was performed on purified and non-purified myoblasts.

As shown in FIGS. 4 A to C and 5, the proportion of BB13 cells cultured in the LOBSFM serum-free medium that expressed detectable levels of desmin diminished progressively during the culture. This reduction in desmin expression was more pronounced for non-purified myoblasts (such as H49 and H51 cells, FIG. 5 B) than for purified myoblasts (BB13 cells, FIG. 5 A). In comparison, most of myoblasts cultured in the STD medium maintained high levels of desmin expression, for an extended culture period (six weeks, FIG. 4 I and FIG. 5). Nevertheless, when transferred in a differentiation medium, both BB13 cells expanded in STD medium or LOBSFM fused and expressed desmin similarly (FIGS. 4 B and E). This result indicates that culture in the LOBSFM medium does not alter the ability of the cells to differentiate to myotubes.

As shown on FIGS. 4 G and H, BB13 cells first cultured in the STD medium fused efficiently when transferred to the differentiation medium (FIGS. 4 G and H). In comparison to cells cultured in the STD medium, BB13 cells first cultured for 25 days in the LOBSFM showed similar, or even higher, ability to form myotubes and fuse (FIG. 7). These results indicate that the LOBSFM medium supports the expansion/

proliferation of myoblasts while maintaining their myogenic potential, even though desmin expression can decrease during culture.

Myoblasts cultured for four weeks in LOBSFM or STD medium exhibited similar proportion of NKH-1 expressing BB13 cells (FIG. 6 A). However, although the expression levels were similar for BB13 cells, it was lower for non-purified H49 cells cultured in LOBSFM (FIG. 6 B).

Without wishing to be bound to theory, Applicant has hypothesized that this loss in desmin expression during culture could be caused by two phenomena: 1) the medium supports the proliferation of cells other than myoblasts or 2) certain cells can dedifferentiate during the culture. As indicated above, BB13 cells form a rather uniform population and express initially 95-100% desmin positive. Although phenomenon 1 can be observed during expansion of unpurified biopsies, it would be most unlikely in this case when culturing BB13 cells. Rather, it is most likely that BB13 cells dedifferentiate during culture in the LOBSFM. This dedifferentiation phenomenon has already been reported for other culture. For example, it was observed that the CNTF (ciliary neurotrophic factor) allowed myoblast clones (desmin<sup>+</sup>) to form multipotent progenitors, having a self-renewal capacity and being able to form several cellular types, in particular neurons, smooth muscles cells and adipocytes. These cells proliferated during more than 20 passages without expressing the muscle regulatory factor (MRF) and it was possible to differentiate them back to the myogenic lineage afterward (Chen et al. 2005). In addition, it is also been shown that IL-1 $\beta$  blocked myogenin expression and the synthesis of several other proteins (Broussard et al. 2004). Finally, it was previously reported that TWEAK decreases not only the expression of myogenin and MyoD, but also of the actin filaments (Girgenrath et al. 2006). Consequently, the reduction of a marker expression during a culture, such as observed here for desmin in LOBSFM-cultured myoblasts, is not necessarily indicative of a loss in myogenesis capacity. The most important test for testing the functionality of myoblasts is a functional fusion in vitro assay to determine the potential of the cells to fuse together to form myotubes or a functional in vivo fusion assay in DMD patients. The added benefit of the in vitro assay is that it can be performed rapidly as a routine assay and is a good indicator of the in vivo situation.

The expression of several genes, specific or not for myoblasts, has also been quantified by RT-PCR as described in Example I. The RNA of P<sub>3</sub> BB13 cells cultured in STD medium for one passage has been isolated with TRIZOL®. The intensity of the RT-PCR product staining on an agarose gel has been listed in Table 9. The results obtained were used to select potential factors to be tested as additives, as well as interpret proliferation assay results previously presented.

TABLE 9

List of genes detected by RT-PCR and classified according to the bands on the agarose gel

Target	Intensity
$\beta$ -actin	+
BMP-R1 $\alpha$	+++
C-MET	++
COL1	+++
DHH	++
EGF	+++
EGF-R	+++
EPOR	++
FGF-1	+++

TABLE 9-continued

List of genes detected by RT-PCR and classified according to the bands on the agarose gel	
Target	Intensity
FGF-5	++
FGFR-1	+++
FGFR-4	++
GAPDH	+++
GM-CSFR $\beta$	+
IGF-1	+
IGF-1R	++
IGF-II	+++
IGF-IIR	+
IHH	+
IL-4R $\alpha$	+
IL-6R	+
IL-11	++
IL-13R $\alpha$ 1	+++
IL-13R $\alpha$ 2	+
IL-15R $\alpha$	+
IL-20R $\beta$	+
MRF4	++
Myf5	++
MyoD	+++
Myostatin	++
NGF	+
p75 (NGF receptor)	++
PDGF-A	+++
PDGFR $\alpha$	+
PTC2 (Hh receptor)	++
sEPOR	++
SSH	+++
TGF $\beta$ -1	+
TGF $\beta$ -R1	+
TGF $\beta$ -2	+++
TGF $\beta$ -3	+++
TGF $\beta$ -R2	+
TGF $\beta$ -R3	++
TNFR-1	+
TrkA (NGF receptor)	+

## Example VI

## Comparison of Initial Seeding Density and Lag Phase for Cells Cultured in Standard and Serum-Free Medium

In order to determine the minimal initial seeding cell density required to avoid a cell culture lag phase, P<sub>4</sub> BB13 cells were trypsinized and inoculated in 6-well plasma treated plates (Sarstedt 83.1839, 2 ml/well) at a concentration of either 2 000, 10 000 or 40 000 cells/ml in STD medium or LOBSFM. Cells were counted on the fifth day of culture. As shown in FIG. 8, the minimal initial cell concentration of 10 000 cell/ml allowed the culture to grow at a rate similar to the higher initial cell density. No statistical difference in the results obtained with the two culture media (STD and LOBSFM) was observed.

## Example VII

## Comparison of a Freeze/Thaw Cycle on the Growth of Cells Cultured in Serum-Free Medium

In order to determine the effect of LOBSFM freezing on cell growth, P<sub>4</sub> BB13 were trypsinized and inoculated in 24-well plasma treated plates (Sarstedt 83.1836, 500  $\mu$ L/well)

at an initial density of 10 000 cells/ml in either fresh STD medium or LOBSFM, or LOBSFM that has been frozen at -20° C. overnight, and then thawed. As shown in FIG. 9, a previously frozen LOBSFM does not significantly impact its capacity to expand myoblasts.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.

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<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 47

acctgagcgt catgtaccg 19

<210> SEQ ID NO 48
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 48

tgtggcacga ggagatgtag 20

<210> SEQ ID NO 49
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 49

gccaaaggtea tccatgacaa c 21

<210> SEQ ID NO 50
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 50

gtccaccacc ctgttgctgt a 21

<210> SEQ ID NO 51
<211> LENGTH: 24
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 51

agcttcctgc tcaagtgttt agag 24

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<210> SEQ ID NO 52  
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<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 52

ttcttccatc tgctgccaga tggt 24

<210> SEQ ID NO 53  
<211> LENGTH: 24  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 53

gtctcctgaa cctgagtaga gaca 24

<210> SEQ ID NO 54  
<211> LENGTH: 24  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 54

aaggggatga caagcagaaa gtcc 24

<210> SEQ ID NO 55  
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<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 55

cttctctctg accagca 17

<210> SEQ ID NO 56  
<211> LENGTH: 17  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 56

acatgggttc ctgagtc 17

<210> SEQ ID NO 57  
<211> LENGTH: 20  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
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<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 57

tggagtggcc tctggttatg 20

<210> SEQ ID NO 58  
<211> LENGTH: 22  
<212> TYPE: DNA  
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<220> FEATURE:  
<223> OTHER INFORMATION: Oligonucleotide

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<400> SEQUENCE: 58

gggaactagg gagacagacg ag

22

<210> SEQ ID NO 59

<211> LENGTH: 24

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 59

ggtgctgaag ctctttcttg ctgc

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<211> LENGTH: 24

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 60

attatgggag gcccaatcct agac

24

<210> SEQ ID NO 61

<211> LENGTH: 22

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 61

ctacactgga ttgatcaact at

22

<210> SEQ ID NO 62

<211> LENGTH: 22

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 62

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22

<210> SEQ ID NO 63

<211> LENGTH: 20

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 63

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<210> SEQ ID NO 64

<211> LENGTH: 20

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 64

cttctgggtc ttgggcatgt

20

<210> SEQ ID NO 65

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<211> LENGTH: 20  
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 <220> FEATURE:  
 <223> OTHER INFORMATION: Oligonucleotide  
  
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 <212> TYPE: DNA  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Oligonucleotide  
  
 <400> SEQUENCE: 66  
  
 caggttcac tttcaccac 19  
  
  
 <210> SEQ ID NO 67  
 <211> LENGTH: 20  
 <212> TYPE: DNA  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Oligonucleotide  
  
 <400> SEQUENCE: 67  
  
 cctggagacg tactgtgcta 20  
  
  
 <210> SEQ ID NO 68  
 <211> LENGTH: 18  
 <212> TYPE: DNA  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Oligonucleotide  
  
 <400> SEQUENCE: 68  
  
 ggactgcttc caggtgtc 18  
  
  
 <210> SEQ ID NO 69  
 <211> LENGTH: 20  
 <212> TYPE: DNA  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Oligonucleotide  
  
 <400> SEQUENCE: 69  
  
 gctgtttgta tgcacgactt 20  
  
  
 <210> SEQ ID NO 70  
 <211> LENGTH: 20  
 <212> TYPE: DNA  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Oligonucleotide  
  
 <400> SEQUENCE: 70  
  
 tgctctggac tctgtgattt 20  
  
  
 <210> SEQ ID NO 71  
 <211> LENGTH: 19  
 <212> TYPE: DNA  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Oligonucleotide  
  
 <400> SEQUENCE: 71



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ctacgccccg ctcacaaag	19
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ctgatgtacc agttggggaa	20
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ggaagccgtg gttatctctg tt	22
 <210> SEQ ID NO 78 <211> LENGTH: 22 <212> TYPE: DNA	

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<213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Oligonucleotide  
  
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 <210> SEQ ID NO 79  
 <211> LENGTH: 23  
 <212> TYPE: DNA  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Oligonucleotide  
  
 <400> SEQUENCE: 79  
  
 atccccacacg ccacattcaa agc 23  
  
  
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 <211> LENGTH: 23  
 <212> TYPE: DNA  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Oligonucleotide  
  
 <400> SEQUENCE: 80  
  
 tgccccacca cgaaatgata aat 23  
  
  
 <210> SEQ ID NO 81  
 <211> LENGTH: 20  
 <212> TYPE: DNA  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Oligonucleotide  
  
 <400> SEQUENCE: 81  
  
 gcccccatct ccctccaagt 20  
  
  
 <210> SEQ ID NO 82  
 <211> LENGTH: 20  
 <212> TYPE: DNA  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Oligonucleotide  
  
 <400> SEQUENCE: 82  
  
 aggggaaggg cgaagagagc 20  
  
  
 <210> SEQ ID NO 83  
 <211> LENGTH: 22  
 <212> TYPE: DNA  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Oligonucleotide  
  
 <400> SEQUENCE: 83  
  
 acccaccaat cacgaaccta ag 22  
  
  
 <210> SEQ ID NO 84  
 <211> LENGTH: 23  
 <212> TYPE: DNA  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Oligonucleotide  
  
 <400> SEQUENCE: 84  
  
 gggtcacattt ctgttaaggt ccc 23

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<210> SEQ ID NO 85  
<211> LENGTH: 25  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 85

actctgtgca ccgagttgac cgtaa

25

<210> SEQ ID NO 86  
<211> LENGTH: 25  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 86

tctcatgata gtcttttagcc ttccc

25

<210> SEQ ID NO 87  
<211> LENGTH: 20  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 87

tgcgtctccg actacatgag

20

<210> SEQ ID NO 88  
<211> LENGTH: 19  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 88

tgactgcata ggtgagatg

19

<210> SEQ ID NO 89  
<211> LENGTH: 21  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 89

tgcctacgtg tatgccatcc c

21

<210> SEQ ID NO 90  
<211> LENGTH: 21  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 90

cttgccctc attctcactg c

21

<210> SEQ ID NO 91  
<211> LENGTH: 20  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:

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<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 91

ccctgaggac acgcagtatt                                20

<210> SEQ ID NO 92
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 92

tgatcaaagg gcctgatagc                                20

<210> SEQ ID NO 93
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 93

atgaactcct tctccacaag cgc                            23

<210> SEQ ID NO 94
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 94

gaagagccct caggctggac tg                             22

<210> SEQ ID NO 95
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 95

cattgccatt gttctgaggt tc                             22

<210> SEQ ID NO 96
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 96

agtagtctgt attgctgatg tc                             22

<210> SEQ ID NO 97
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 97

tctaattggtc agcatcgatc a                             21

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<210> SEQ ID NO 98  
<211> LENGTH: 20  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 98

gtggagatca aaatcaccag 20

<210> SEQ ID NO 99  
<211> LENGTH: 20  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 99

tgctcaaaat ggagacttgg 20

<210> SEQ ID NO 100  
<211> LENGTH: 19  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 100

gagggcccca catatttca 19

<210> SEQ ID NO 101  
<211> LENGTH: 20  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 101

tgagaaccaa gaccagaca 20

<210> SEQ ID NO 102  
<211> LENGTH: 20  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 102

tcattggcttt gtagatgcct 20

<210> SEQ ID NO 103  
<211> LENGTH: 21  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 103

atgaactgtg tttgccgcct g 21

<210> SEQ ID NO 104  
<211> LENGTH: 21  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Oligonucleotide

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<400> SEQUENCE: 104

gagctgtaga gctcccagtg c

21

&lt;210&gt; SEQ ID NO 105

&lt;211&gt; LENGTH: 20

&lt;212&gt; TYPE: DNA

&lt;213&gt; ORGANISM: Artificial Sequence

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: Oligonucleotide

&lt;400&gt; SEQUENCE: 105

tcacaaagga ggcgaggttc

20

&lt;210&gt; SEQ ID NO 106

&lt;211&gt; LENGTH: 20

&lt;212&gt; TYPE: DNA

&lt;213&gt; ORGANISM: Artificial Sequence

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: Oligonucleotide

&lt;400&gt; SEQUENCE: 106

tgaacggcat ccaccatgac

20

&lt;210&gt; SEQ ID NO 107

&lt;211&gt; LENGTH: 20

&lt;212&gt; TYPE: DNA

&lt;213&gt; ORGANISM: Artificial Sequence

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: Oligonucleotide

&lt;400&gt; SEQUENCE: 107

aaggaatacc agtcccgaca

20

&lt;210&gt; SEQ ID NO 108

&lt;211&gt; LENGTH: 21

&lt;212&gt; TYPE: DNA

&lt;213&gt; ORGANISM: Artificial Sequence

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: Oligonucleotide

&lt;400&gt; SEQUENCE: 108

accagggaac catgaaacaa g

21

&lt;210&gt; SEQ ID NO 109

&lt;211&gt; LENGTH: 20

&lt;212&gt; TYPE: DNA

&lt;213&gt; ORGANISM: Artificial Sequence

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: Oligonucleotide

&lt;400&gt; SEQUENCE: 109

gtgaaacatg gaagaccatc

20

&lt;210&gt; SEQ ID NO 110

&lt;211&gt; LENGTH: 24

&lt;212&gt; TYPE: DNA

&lt;213&gt; ORGANISM: Artificial Sequence

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: Oligonucleotide

&lt;400&gt; SEQUENCE: 110

gtgaaataac tggatctgat aggc

24

&lt;210&gt; SEQ ID NO 111

&lt;211&gt; LENGTH: 21

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<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Oligonucleotide  
  
<400> SEQUENCE: 111  
  
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<210> SEQ ID NO 112  
<211> LENGTH: 21  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Oligonucleotide  
  
<400> SEQUENCE: 112  
  
gtgaacatca ctttcgtat a 21  
  
<210> SEQ ID NO 113  
<211> LENGTH: 18  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Oligonucleotide  
  
<400> SEQUENCE: 113  
  
cgccaggtgt gtatccac 18  
  
<210> SEQ ID NO 114  
<211> LENGTH: 20  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Oligonucleotide  
  
<400> SEQUENCE: 114  
  
gtttgccttg acttgaggta 20  
  
<210> SEQ ID NO 115  
<211> LENGTH: 19  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Oligonucleotide  
  
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tccccagttg attggaaga 19  
  
<210> SEQ ID NO 116  
<211> LENGTH: 21  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Oligonucleotide  
  
<400> SEQUENCE: 116  
  
agtcaaacct tccttcttgg a 21  
  
<210> SEQ ID NO 117  
<211> LENGTH: 22  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Oligonucleotide  
  
<400> SEQUENCE: 117

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gcttcaccct gtggaacgaa tc 22

<210> SEQ ID NO 118  
 <211> LENGTH: 21  
 <212> TYPE: DNA  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Oligonucleotide

&lt;400&gt; SEQUENCE: 118

ggagatgccc gtgatgaacc a 21

<210> SEQ ID NO 119  
 <211> LENGTH: 21  
 <212> TYPE: DNA  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Oligonucleotide

&lt;400&gt; SEQUENCE: 119

acctggaatc agattacttt g 21

<210> SEQ ID NO 120  
 <211> LENGTH: 19  
 <212> TYPE: DNA  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Oligonucleotide

&lt;400&gt; SEQUENCE: 120

ccatacctct aggctggt 19

<210> SEQ ID NO 121  
 <211> LENGTH: 22  
 <212> TYPE: DNA  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Oligonucleotide

&lt;400&gt; SEQUENCE: 121

ccttcagcca gacagaatgt gt 22

<210> SEQ ID NO 122  
 <211> LENGTH: 19  
 <212> TYPE: DNA  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Oligonucleotide

&lt;400&gt; SEQUENCE: 122

gcaaacaggg ccagtacca 19

<210> SEQ ID NO 123  
 <211> LENGTH: 25  
 <212> TYPE: DNA  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Oligonucleotide

&lt;400&gt; SEQUENCE: 123

tgtggaggct atggaagaag atatg 25

<210> SEQ ID NO 124  
 <211> LENGTH: 20  
 <212> TYPE: DNA  
 <213> ORGANISM: Artificial Sequence



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<220> FEATURE:  
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 124

gtgcacccac ccatttcttg 20

<210> SEQ ID NO 125  
<211> LENGTH: 24  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 125

acaacacaga cgttcgtctc attg 24

<210> SEQ ID NO 126  
<211> LENGTH: 23  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 126

gaacagcact tcttcaaggg tga 23

<210> SEQ ID NO 127  
<211> LENGTH: 25  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 127

cctgagctac agatatgtca ccaag 25

<210> SEQ ID NO 128  
<211> LENGTH: 20  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 128

ggctggaaag tcaggactcg 20

<210> SEQ ID NO 129  
<211> LENGTH: 24  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 129

gccatacgcc acccatgtca caac 24

<210> SEQ ID NO 130  
<211> LENGTH: 24  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 130

gttggggcca catagcttgt ccag 24

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<210> SEQ ID NO 131  
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<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 131

gtggcagtgg ctgtcattgt tggagtgg 29

<210> SEQ ID NO 132  
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<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 132

tcattctgcgg ctgggtttgg tattttcttc 29

<210> SEQ ID NO 133  
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<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 133

ttgggagtgg acacctgcag tct 23

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<400> SEQUENCE: 134

ccttggtgaa gcagctcttc agcc 24

<210> SEQ ID NO 135  
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<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 135

gctcgtgata acggctaagg aa 22

<210> SEQ ID NO 136  
<211> LENGTH: 20  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
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<400> SEQUENCE: 136

cgatggaaga aaggcatcga 20

<210> SEQ ID NO 137  
<211> LENGTH: 26  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
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<210> SEQ ID NO 138  
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<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 138  
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<210> SEQ ID NO 139  
<211> LENGTH: 18  
<212> TYPE: DNA  
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<220> FEATURE:  
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<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 141  
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<223> OTHER INFORMATION: Oligonucleotide

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tcacgctcct cctgggtg 18

<210> SEQ ID NO 143  
<211> LENGTH: 20  
<212> TYPE: DNA  
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<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 143  
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<210> SEQ ID NO 144

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<211> LENGTH: 20  
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<400> SEQUENCE: 144

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<400> SEQUENCE: 145

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<210> SEQ ID NO 146  
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<400> SEQUENCE: 146

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<210> SEQ ID NO 147  
<211> LENGTH: 20  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
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<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 147

agctttctat cctggccaca 20

<210> SEQ ID NO 148  
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<212> TYPE: DNA  
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<223> OTHER INFORMATION: Oligonucleotide

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gacctgagt gtctgcagct t 21

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<220> FEATURE:  
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<400> SEQUENCE: 149

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<210> SEQ ID NO 150  
<211> LENGTH: 20  
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<400> SEQUENCE: 150

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<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 157

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<210> SEQ ID NO 158
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 158

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<210> SEQ ID NO 159
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<212> TYPE: DNA
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<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 159

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<210> SEQ ID NO 160
<211> LENGTH: 20
<212> TYPE: DNA
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<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 160

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<210> SEQ ID NO 161
<211> LENGTH: 20
<212> TYPE: DNA
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<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 161

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<210> SEQ ID NO 162
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<212> TYPE: DNA
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<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 162

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<210> SEQ ID NO 163
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<212> TYPE: DNA
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<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 163

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<210> SEQ ID NO 164  
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<220> FEATURE:  
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 164

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<210> SEQ ID NO 165  
<211> LENGTH: 21  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 165

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<210> SEQ ID NO 166  
<211> LENGTH: 20  
<212> TYPE: DNA  
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<220> FEATURE:  
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 166

aggttgctca gcacacactc 20

<210> SEQ ID NO 167  
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<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 167

cggagcgagg aagggaag 19

<210> SEQ ID NO 168  
<211> LENGTH: 23  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 168

ttggggataa actgcttgta ggc 23

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<211> LENGTH: 20  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 169

accaactatt gcttcagetc 20

<210> SEQ ID NO 170  
<211> LENGTH: 18  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
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<223> OTHER INFORMATION: Oligonucleotide

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<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 171

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<210> SEQ ID NO 172
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<212> TYPE: DNA
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<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 172

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<210> SEQ ID NO 173
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<212> TYPE: DNA
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<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 173

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<210> SEQ ID NO 174
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<212> TYPE: DNA
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<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 174

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<210> SEQ ID NO 175
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<212> TYPE: DNA
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<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 175

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<210> SEQ ID NO 176
<211> LENGTH: 20
<212> TYPE: DNA
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<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 176

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<210> SEQ ID NO 177  
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<220> FEATURE:  
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<400> SEQUENCE: 177

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<210> SEQ ID NO 178  
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<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
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<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 178

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<212> TYPE: DNA  
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<400> SEQUENCE: 179

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<210> SEQ ID NO 180  
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<212> TYPE: DNA  
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<400> SEQUENCE: 180

actgctgttt tccgaggc 18

<210> SEQ ID NO 181  
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<212> TYPE: DNA  
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<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 181

tcagcctctt ctcttcctg 20

<210> SEQ ID NO 182  
<211> LENGTH: 20  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 182

tgaagaggac ctgggagtag 20

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<210> SEQ ID NO 183  
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<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 183

accaagtgcc acaaaggaac 20

<210> SEQ ID NO 184  
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<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 184

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<210> SEQ ID NO 185  
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<212> TYPE: DNA  
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<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 185

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<210> SEQ ID NO 186  
<211> LENGTH: 20  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
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<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 186

caccagggga agaactctgag 20

<210> SEQ ID NO 187  
<211> LENGTH: 21  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 187

caatgtcacc agtgacctca a 21

<210> SEQ ID NO 188  
<211> LENGTH: 20  
<212> TYPE: DNA  
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<220> FEATURE:  
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 188

tgaactcgaa agggttgtcc 20

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What is claimed is:

1. A culture medium for a cell of a myogenic lineage, said culture medium comprising:

a basal medium;

a growth factor that is at least one of a fibroblast growth factor (FGF), an epidermal growth factor (EGF) and an insulin-like growth factor (IGF), wherein the growth factor has a concentration in the culture medium that is between about 1 ng/mL and about 100 ng/mL; and an interleukin that is at least one of IL-1, IL-6, IL-8, IL-18 and IL-33, wherein the interleukin has a concentration in the culture medium that is between about 0.1 ng/mL and about 100 ng/mL;

wherein the culture medium is free of serum and allows proliferation of the cell of the myogenic lineage at a similar rate of proliferation to the rate of proliferation of another cell of the myogenic lineage that is cultured in a standard medium containing serum.

2. The culture medium of claim 1, wherein the cell of the myogenic lineage is at least one of a muscular stem cell, a myoblast and a myoblast-derived cell.

3. The culture medium of claim 1, wherein the basal medium is at least one of Dulbecco's modified Eagles's medium (DMEM), advanced DMEM, Ham's F10, Ham's F12, Iscove's modified Dulbecco's medium, neurobasal medium, RPMI 1640 and MCDB120 medium.

4. The culture medium of claim 1, further comprising a supplement.

5. The culture medium of claim 4, wherein the supplement is at least one of:

a combination of insulin, transferrin and selenite (ITS); B27<sup>TM</sup>;

a combination of dexamethasone, insulin, EGF, fetuin and albumin; and

a combination of dexamethasone, bFGF, albumin and insulin.

6. The culture medium of claim 1, further comprising a lipid.

7. The culture medium of claim 6, wherein the lipid is at least one of arachidonic acid, cholesterol, DL- $\alpha$ -tocopherol acetate, linoleic acid, linolenic acid, myristic acid, oleic acid, palmitoleic acid, palmitic acid and stearic acid.

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8. The culture medium of claim 1, wherein the growth factor is a combination of FGF, EGF and IGF.

9. The culture medium of claim 8, wherein FGF is a combination of bFGF and FGF4.

10. The culture medium of claim 8, wherein IGF is IGF-1.

11. The culture medium of claim 1, wherein the interleukin is IL-1.

12. The culture medium of claim 11, wherein the IL-1 is a combination of IL-1 $\alpha$  and IL-1 $\beta$ .

13. An in vitro method of culturing a cell of a myogenic lineage, said method comprising contacting the cell of the myogenic lineage with the culture medium of claim 1, thereby culturing said cell.

14. The in vitro method of claim 13, wherein the cell of the myogenic lineage is at least one of a muscular stem cell, a myoblast and a myoblast-derived cell.

15. The in vitro method of claim 14, wherein the myoblast-derived cell is at least one of a muscle cell, a satellite cell and a myocyte.

16. The in vitro method of claim 13, wherein the method is performed for at least an hour.

17. The in vitro method of claim 13, wherein the method is performed for at least a day.

18. The in vitro method of claim 13, wherein the method is performed for at least a week.

19. The in vitro method of claim 13, wherein said method reduces a lag phase of the cell of the myogenic lineage that is cultured in said culture medium of claim 1, with respect to the lag phase of a cell of the myogenic lineage that is cultured in another serum-free media.

20. The in vitro method of claim 13, wherein said method enables long term expansion of the cell of the myogenic lineage.

21. The in vitro method of claim 13, wherein an initial concentration of the cell of the myogenic lineage in the culture medium is 10,000 cells/mL.

22. The in vitro method of claim 13, wherein the method enables the cell of the myogenic lineage to retain its ability to form a myotube.

\* \* \* \* \*